CELL-RESPONSIVE SYNTHETIC BIOMATERIALS

FORMED *IN SITU*

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We all agree that your theory is crazy, but is it crazy enough?
Niels Bohr (1885-1962)

for Patrick

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ABSTRACT

Two-way communication between cells and their extracellular matrices plays an important role in establishing and maintaining cell and tissue morphology. Synthetic materials for tissue engineering have so far not been able to mimic these interactions. This work describes a new class of synthetic biomaterials that display the essential characteristics of naturally occurring matrices. The materials can be formed in contact with cells and tissues to create covalently cross-linked hydrogels which are effectively nonporous on the length scale of a cell process. The otherwise nonadhesive gels can be rendered specifically cell-adhesive by incorporation of ligands for cell surface adhesion molecules, such as integrins. The materials are degradable by plasmin, a proteolytic enzyme used by cells during proliferation and migration in natural matrices. Furthermore, the materials can be designed to sequester heparin-binding growth factors.

The materials consist primarily of poly(ethylene glycol) which is cross-linked *in situ* by selective conjugate addition reactions of its termini, activated with conjugate acceptors such as vinylsulfones, with peptides containing three or more cysteine residues in the form of reduced thiols. The cross-linking peptides are designed such that they are hydrolyzed by plasmin. A simple cell migration assay was developed to evaluate the material design parameters. Human fibroblasts were observed to migrate out of microliter fibrin clots into the synthetic materials in a manner dependent on the type of material and the culture conditions. Inhibiting fibrinolysis with a plasmin-insensitive substrate or with a plasmin inhibitor, ε-amino-n-caproic acid, inhibited migration. Adding growth factors, FGF-2 or PDGF-BB, that upregulate plasminogen activation increased the rate of cell migration. Decreasing the amount of RGD within the materials decreased the rate and extent of cell

migration in a concentration-dependent manner. Replacing the RGD peptide with an RDG peptide decreased the amount of outgrowth.

In a critical size calvarial defect in the rat, the synthetic materials were formed in the presence of an osteoinductive protein, rhBMP-2, and supported *de novo* bone formation. Materials containing a heparin-based growth factor delivery system promoted significantly more bone than materials without the delivery system. After three weeks, 94% of the defect area was covered with new bone, and the opacity of the new bone was 84% of that of neighboring, uninjured bone.

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ABBREVIATIONS

aa	acetamide
Ac	acetyl
Ala	alanine
Arg	arginine
Arg-	the peptide bond on the carboxy-side of arginine
Asn	asparagine
Asp	aspartic acid
ATIII	antithrombin III
BMP	bone morphogenetic protein
C, Cys	cysteine
C2	peptide GCYKNRDCG
C3	peptide GCYKNRCYKNRCG
C4	peptide GCYKNRCYKNRCYKNRCG
cDNA	complementary deoxyribonucleic acid
d	days
D	notation for D isomers of amino acids
D	aspartic acid
***D*C2**	peptide GCY-DLys-N-DArg-DCG
***D*C3**	peptide GCY-DLys-N-DArg-CY-DLys-N-DArg-CG
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
E8	embryonic day 8
EACA	ε-amino-n-caproic acid
ECM	extracellular matrix
eq	molar equivalent
FBS	fetal bovine serum
FGF	fibroblast growth factor
G, Gly	glycine
GAG	glycosaminoglycan
Gln	glutamine
Glu	glutamic acid
GRAVY	grand average hyropathicity index
His	histidine
hr	hours
HEPES BS	N-[2-hydroxyethyl]piperazine-N'-[4-butanesulfonic acid] buffered saline
Ile	isoleucine
K	lysine
kD	kilodalton
K_m	Michaelis constant
L	liters
Leu	leucine
Lys	lysine
Lys-	the amide bond on the carboxy-side of lysine
Met	methionine

min	minutes
MMP	matrix metalloproteinase
MW	molecular weight
N	asparagine
OP-1	osteogenic protein-1, BMP-7
P1, P2, P3	amino acids 1, 2, 3 positions amino-terminal to an enzymatic cleavage site
P1', P2', P3'	amino acids 1, 2, 3 positions carboxy-terminal to an enzymatic cleavage site
Pa	Pascal, unit of pressure
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PEG	poly(ethylene glycol)
PGA	poly(glycolic acid)
Phe	phenylalanine
pI	isoelectric point
PLA	poly(lactic acid)
PLGA	poly(lactic-*co*-glycolic acid)
pNA	para-nitroanilide
Pro	proline
Q	glutamine
R	arginine
RDG	arginine-aspartic acid-glycine-containing peptide
RGD	arginine-glycine-aspartic acid-containing peptide
rh	recombinant human
s	seconds
Ser	serine
TBS	Tris buffered saline
TGF-β	transforming growth factor
Thr	threonine
tPA	tissue-type plasminogen activator
Trp	tryptophan
Tyr	tyrosine
U	units of enzyme activity, as defined by the manufacturer
uPA	urokinase plasminogen activator
Val	valine
V_{max}	maximal reaction rate
VS	vinylsulfone
wk	weeks
Y	tyrosine

CHAPTER 1

INTRODUCTION

DEMAND FOR BIOMATERIALS

"The loss or failure of an organ or tissue is one of the most frequent, devastating, and costly problems in human health care."[1] Also, as humans are living longer on average, the demands for tissue and organ repair and replacement are growing.[2] The demands for "spare parts" include skin, bone, cartilage, blood vessels, pancreas, kidney, liver, nerve, and dental reconstructions, among others, and require more than eight million surgical procedures annually in the United States alone.[1] Physicians do their best to meet tissue and organ malformations, disease, and injuries by using surgical reconstructions, transplants, implants, and extracorporeal mechanical devices, such as dialysis machines. However, these solutions are often imperfect, and better ones are continually sought. As biologic knowledge expands and is combined with engineering, material science, and other expertise, tissue engineering solutions are being developed to restore tissue and organ function with biological substitutes – cell-based therapies, biomaterials, and their combination.

DEFINITION OF A BIOMATERIAL

A biomaterial is any material in contact with a biological system, e.g., cells, tissues, or organs. A successful biomaterial leads to the desired outcome, whether it be passive or active, so success can only be defined for a particular material in a specific application. Biomaterials may be biologically derived from animals, plants, or culture of bacteria, yeast, or plant or animal cells, or they may be formed by synthetic chemical methods. Both

categories are subject for discussion in this introduction since the goals of the work presented in this thesis were to design, synthesize, and evaluate the design principles of a new class of synthetic materials which mimic biological materials to a sufficient degree to support three-dimensional cell culture, allow cell migration and remodeling within the materials, and have potential for application in therapeutic tissue engineering applications.

NATURE'S BIOMATERIALS

Fibrin

Fibrin is the natural material for wound healing. It is found at sites of vascular damage and is the insoluble result of the cross-linking of soluble, circulating fibrinogen (340 kDa, 2.5 mg/ml) in the final steps of the coagulation cascade. Thrombin cleavage of two fibrinopeptides from fibrinogen initiates fibrin formation. It is followed by half-staggered self-assembly of the resulting fibrin monomers into double stranded protofibrils, fiber formation and aggregation, and finally covalent cross-linking of fibrin monomers by a transglutaminase, factor XIIIa, which is also activated by thrombin. Factor XIIIa forms lysine-glutamine cross-links in the γ and α chains of fibrin. Fibrin(ogen) interacts with a number of proteins, including plasminogen, plasminogen activator, fibronectin, antithrombin III (ATIII) and α2-plasmin inhibitor, the latter three of which are cross-linked to fibrin by factor XIIIa. [3]

Fibrin prevents further blood loss and is then a substrate for cell attachment. Leukocytes, monocytes,[4] and platelets [5] bind by integrins to its γ chains. Platelet adhesion can be inhibited by soluble RGD-containing peptides;[6] however, it is not clear if physiologic platelet adhesion is RGD-mediated by one of the two RGD domains in fibrin. Both RGD

domains reside on the α chain of fibrin. One, α572-575, interacts with the $\alpha_v\beta_3$ integrin expressed by endothelial cells and fibroblasts.[7] The other, α95-98, is structurally unfavorable for cell interaction.[8]

During clot resolution, fibrin is degraded by proteolytic enzymes. It can be degraded by plasmin, elastase, and cathepsin G;[9] however, the primary fibrinolytic enzyme is plasmin. Dissolution of fibrin clots in physiological conditions occurs in a highly regulated manner. Fibrinolysis yields a complex pattern of degradation products smaller than 260 kDa, some of which are biologically active, enhancing vascular permeability or further promoting anti-coagulant activity.

Fibrin has had long and broad clinical use, primarily as a hemostatic agent, sealant, and mechanism of drug delivery[10, 11] but also as a tool for tissue engineering, e.g., as a means to improve patency of vascular grafts.[12] However, fibrin's inherent capacity for wound healing is limited; fibrin is optimized to stop bleeding and form scar tissue rather than encourage functional recovery, e.g., nerve regeneration over long gaps. In cases where scarless healing or functional recovery is desired, then fibrin can be enhanced[13, 14, 15] or other materials must be applied. For clinical applications, fibrin(ogen)'s biggest disadvantage is that it is a blood product. While it can be prepared from autologous blood in some clinical settings,[10] in most cases it is supplied by pooled, partially purified, allogenic sources which may transmit diseases such as HIV and hepatitis.

Extracellular matrix

The extracellular matrix (ECM) is the natural scaffold for tissues. Its composition and the roles it plays are both extremely diverse and extremely complex. It is a space-filler,

and some cell types, such as fibroblasts and chondrocytes of the connective tissue, are completely surrounded by ECM. Thin polarized ECM, called basement membrane or basal lamina, delineates borders between tissue types, such as muscle and nerve, and provides the substrate for cells such as epithelial and endothelial cells that are in contact with a single surface.[16] ECM provides appropriate mechanical support to cells and tissues, and it influences cell behavior by presenting ligands for cell adhesion, important because most cells are anchorage dependent, and sequestering growth factors that regulate cell survival, proliferation, differentiation, and migration.[17] ECM also controls trafficking of molecules and cells.

Collagen

Collagen is a major component of ECM, especially collagen type I in skin, tendons, ligaments, bone, and internal organs, and accounts for approximately 30% of the total protein in vertebrates. The primary role of collagen is to provide mechanical strength to tissues and organs, but it is also important for cell attachment, differentiation, and migration. Collagen's roles are related to its triple-helical structure and ability to form stable higher order structures. Collagen is formed from three left-handed helical α chains (2° structure). Each chain is more than one thousand amino acid residues long and composed of Gly-X-Y repeats (1° structure). X and Y can be any amino acid but are most often proline and hydroxyproline, respectively, which are, along with the small molecule glycine, important for collagen's self-assembly and helical stability. The three α chains twist around each other like a rope (3° structure) to form collagen molecules (300 kDa; type I, II, etc.). The collagen molecules are right-handed triple-helices approximately 300 nm in length, and 1.5 nm in

diameter, making collagen highly viscous in solution. Collagen molecules aggregate into systematically staggered arrays (4° structure) which form higher order fibrillar structures. Collagen molecules are stabilized by hydrogen bonds resulting from the presence of imino acids, hyroxyproline and hydroxylysine. Collagen molecules and fibrils are further stabilized by molecular cross-links, initiated by the action of lysyl oxidase followed by chemical condensation and oxidation-reduction reactions to yield stable cross-links. Collagen's peptide bonds are well buried within the structure making undenatured collagen resistant to many proteases, with the exception of collagenases.[18, 19] And in total, collagen post-translational modification and later degradation requires the activity of at least ten enzymes.[20]

Different combinations of α chains give rise to at least fifteen types of collagens in vertebrates, each having tissue-specific distribution and specialized biological function. For example, Type I fibrillar collagen is the most abundant collagen type found in adult connective tissues such as skin, tendon, and bone. Type II fibrillar cartilage is unique to cartilage and contains extensive post-translational modifications of its lysine residues. Blood vessels contain predominantly collagen type III. Type IV non-fibrillar network-forming collagen is only affiliated with basement membranes. Type X short-chain collagen is synthesized by chondrocytes during the process of endochondral bone formation. Long chain type VII collagen is uniquely located in subepithelial structures to stabilize epithelial basement membrane.

For millenia, collagen has been used as a suture material. Since the 1970s, when processing technologies improved and clinical grade collagens became more widely available, collagen has been applied more broadly in the clinic. It has been used to replace ligaments, tendons, other soft tissues, and as wound dressings. It has always been animal

derived, predominantly from bovine skin, and therefore carries the risk of immunogenicity and pathogen transmission. With effective removal of collagen's telopeptides with papain, collagen's highly conserved triple helical domains do not usually illicit immunological responses. But as a protein, collagen is limited in processibility within the constraints of maintaining its bioactivity. Following extraction and purification, it requires exogenous chemical cross-linking for stability. However, stable cross-links often decrease the enzymatic degradability of collagen.[21, 18, 22]

Elastin

Elastin provides tissues and organs, such as blood vessels, lungs, and skin, with flexibility and the ability to withstand stretch. It is a highly hydrophobic protein in which 70% of its amino acid residues are nonpolar and a small percent are acidic and basic residues which contribute to cross-linking. The unique hydrophobic structure of elastin requires specialized cell synthesis, post-translational modifications, packaging, and transport before elastin can be assembled into fibers. The fibers are a random orientation of cross-linked chains. Stretching causes alignment and ordering of the fibers and creates a disfavored lower entropy state. Upon removal of the stretch force, elastic recoil spontaneously occurs to return the elastin to its high entropy disordered state. Also, in the relaxed state, the side chains of elastin's hydrophobic residues are buried such that glycine and peptide bonds are exposed to water instead. Stretching exposes the hydrophobic side chains to water giving another large negative entropic change and another driving force for elastic recoil upon withdrawal of the stretching force.

Elastin is primarily a structural protein and is otherwise relatively inert; biological recognition of elastin, e.g., by cell adhesion and degradation, occurs at low rates compared to other molecules of ECM. Elastin is synthesized by fibroblasts and some smooth muscle cells, which have low elastase activity; MMPs (72 kDa and 92 kDa) associated with fibroblasts, smooth muscle cells, and macrophages can degrade elastin in some cases. Elastase activity is primarily associated with inflammatory cells and some pathogenic bacteria which can bind elastin. There are no integrin interactions with elastin, but elastin binding receptors, one being the 67 kDa laminin receptor, exist and recognize VGVAPG repeats.[23]

Fibronectin

Fibronectins (520 kDa) are heterodimeric glycoproteins circulating in the blood of all vertebrate species and initially referred to as "cold insoluble globulins" for their poor solubility at low temperatures. They are found in many tissue types, and, after collagens, fibronectins are the main components of connective tissues. Fibronectins are also the second most abundant protein in clots, after fibrin to which they are covalently cross-linked by a glutamine residue near the fibronectin amino-terminus.[24] Fibronectins are also found as dimers and other multimers attached to the surfaces of cells such as fibroblasts, endothelial cells, and platelets. In all of these settings, fibronectins play important roles in cell surface interactions, mediating adhesion, migration, and differentiation. Some of these functions have been isolated to distinct and relatively small domains which are sufficient for binding cell surface receptors and inducing signaling cascades. These domains include the ubiquitous RGD sequence as well as others.[25]

Laminin

Laminin is a large (≈900 kDa) extracellular heterotrimeric glycoprotein found in basal lamina. In particular, laminin is one of the first ECM proteins expressed in a developing embryo and stimulates growth and regeneration of neuronal processes. Homologues to its three subunits exist and give rise to more than eleven laminin isoforms, of which laminin-1 has been the most investigated. The laminins have a distinctive cruciform structure. The carboxy-terminal region of the long arm promotes cell attachment and neurite outgrowth and contains the RNIAEIIKDI and IKVAV domains thought to contribute to these laminin activities. Other cell-interactive regions, such as YIGSR and PDGSR are located on the short arms and near their intersection with the long arm. At least seven integrins bind laminin; binding is RGD independent because the RGD domain in laminins is masked. Some integrins, such as $\alpha_6\beta_1$, bind in a carbohydrate-dependent manner, and this seems to be the major laminin binding mechanism of adhering cells. A 67 kDa protein laminin receptor has both peptide and carbohydrate recognition mechanisms for laminin.[25, 26, 27] Laminin also has binding domains for ECM molecules such as collagen and heparan sulfate.

Proteoglycans

Proteoglycans are a heterogeneous class of complex macromolecules consisting of a core protein backbone with long, covalently grafted glycosaminoclycan (GAG) molecules. GAGs are linear polymers of repeating disaccharides where one saccharide unit is a uronic acid and the other a hexosamine, sulfated or acetylated to different extents in different GAGs. Due to their large content of sulfate and carboxy groups, GAGs are highly negatively

charged. Proteoglycans are found intracellularly, on the cell surface, and in the ECM. The core proteins include serglycin, decorin which decorates collagen fibrils, syndecan, and aggrecan which aggregates and is an important structural component of cartilage. The four classes of GAGs are: hyaluronic acid; chondroitin sulfate and dermatan sulfate; keratan sulfate; and heparin and heparan sulfate. Molecular weights of proteoglycans can reach millions of daltons, and even a single molecule of hyaluronic acid, which is unique in that it is not covalently bound to a core protein, can have a molecular weight of as much as 10×10^6 Da. Due to its size and polyanionic character at physiological conditions, hyaluronic acid occupies a large volume in solution and can form elastic networks by entanglements. During development, hyaluronic acid promotes cell proliferation and migration. In the adult, it is important, for example, in the vitreous humor of the eye. Chondroitin sulfate and heparan sulfate proteoglycans are important in basement membranes where they control permeability of basement membranes, e.g., as a selective filter in the kidney. Heparan sulfate also binds proteins, such as growth factors, and cells.[28] Also, GAGs are relatively inflexible; their inflexibility along with their hydrophilic natures causes them to occupy a large volume per mass and contributes to the gel-like character of the ECM. The turgor pressure GAGs generate by influx of cations and water helps the ECM withstand compressive forces, e.g., in joints.

GROWTH FACTORS

Another important function of the ECM and specifically proteoglycans is to bind and sequester growth factors. This binding is often mediated by heparin in the ECM. Heparin is highly sulfated and has the largest negative charge of naturally occurring polymers.[29]

Therefore, many polypeptide growth factors electrostatically bind heparin with high affinity. Binding of growth factors to heparin protects them from enzymatic degradation and in some cases, e.g., fibroblast growth factors, increases the biological activity of the growth factors through conformational changes which optimize receptor binding, receptor dimerization, and subsequent initiation of signaling cascades. Other growth factors, such as TGF-β, bind to the core protein of GAGs, such as decorin, in which conformation growth factor activity is inhibited. This sequestering of growth factors and localized growth factor activity is important because growth factors are potent modulators of cell behavior, such as survival, proliferation, differentiation, and migration.

INTEGRINS

Since most cells, not including circulating cells, are anchorage dependent, adhesion receptors are one of the most important classes of cell surface molecules. Nonjunctional adhesion mechanisms include cadherins, which engage in homophilic binding in cell-cell interactions, selectins, cell adhesion molecules of the immunoglobulin family, transmembrane proteoglycans, and integrins. Integrins mediate cell-cell interactions and are the principle receptors for extracellular molecules. They are expressed ubiquitously; most cells express several different types of integrins. Integrins are noncovalently associated transmembrane glycoprotein heterodimers of α and β subunits. The extracellular domain is large with ligand binding and cation binding sites. The intracellular domain is associated closely with components of the cytoskeleton or with second messengers such as tyrosine kinases or G proteins. Integrins are important communicators that transmit information bi-directionally across the cell membrane and are important regulators of cell adhesion and

migration. While they bind their ligands with relatively low affinity, integrins are found in high concentrations on the cell surface; this allows cells to explore their environments without losing substrate attachment completely. In many cases, integrin binding domains in proteins can be localized to relatively small peptide sequences of less than 10 or 20 amino acid residues. The RGD peptide was the first such small peptide binding domain to be identified.[30] It and other sequences, as well as their use in biomaterials, are well documented.[31, 32]

Following binding of an integrin to an extracellular ligand, rapid linkage of the receptor to the actin cytoskeleton occurs. Inside the cell, molecular motors generate forces on the cytoskeleton, thus on its newly associated receptor-ligand complexes, and ultimately on the matrix to which the ligands are bound. As a result, the integrin-ligand complexes are driven toward the rear of the cell, disappearing after reaching a certain distance from the leading edge, and the cell is propelled forwards.[33]

Integrin function can be regulated by molecules other than integrin ligands, e.g., interactions of integrins with other cell-surface receptors. For example, growth factors such as FGFs can affect integrin behavior due to interactions between integrins and FGF receptors. Similarly, plasminogen activators can induce intracellular signaling through integrins due to interactions between plasminogen activator receptors (uPAR) with integrins.[34]

ENDOGENOUS MECHANISMS OF MATRIX DEGRADATION

Matrix degradation is primarily mediated by proteolytic enzymes; glycosaminoglycan-degrading enzymes, such as heparatinases, play a relatively minor role. Proteolytic enzymes of five proteinase (endopeptidase) classes are responsible for degrading

proteins at central peptide bonds. These are serine proteinases, cysteine proteinases, aspartic proteinases, metalloproteinases, and a subclass with unknown mechanism. All classes are involved in tissue remodeling. Serine proteases are central to hemostasis and resolution of hemostasis (fibrinolysis); they can also be stored in leukocyte and mast cell granules and released in inflammatory and allergic responses and are partially responsible for processing of other proteins, such as growth factors and hormones. Serine proteases and metalloproteinases are the neutral proteinases used by cells in migration. The acidic proteases, cysteine and aspartic proteases, are particularly important in digestion and degradation of proteins intracellularly.[35] Cleavage of proteins is a fairly specific and carefully regulated process. Protein degradation by a particular enzyme rarely leads to more than 10-15 cleavages per molecule.[36]

Plasminogen

Plasminogen (92 kDa, 2 μM circulating concentration) is present in the extravascular space of most tissues; it is made by a number tissues, but is synthesized predominantly in the liver. It is a single peptide chain of 791 amino acid residues and 24 disulfide bonds. It contains an amino-terminal pre-activation peptide, a carboxy-terminal protease domain, and in between, five kringle domains which can bind fibrin, α2-antiplasmin, and other molecules. Each kringle domain contains approximately 80 amino acids and 3 disulfide bonds and is shaped like a Danish breakfast role from which it obtained its name. Glu-terminated plasminogen (Glu-plasminogen) circulates with a half-life of 2.2 days. Proteolytic cleavage, e.g., by plasmin, of Lys77- in the pre-activation domain of plasminogen gives Lys-plasminogen with a circulating half-life of 0.8 days. Glu-plasminogen undergoes a

conformational change upon becoming Lys-plasminogen such that Lys-plasminogen is more readily activated by plasminogen activators and binds fibrinogen with higher affinity. Also, plasminogen can be localized to the cell surface by its interactions with plasminogen binding sites. These sites are heterogeneous and ubiquitous receptors found in high density on cell surfaces and bind plasminogen predominantly through its lysine binding sites in its kringle domains. Plasminogen can be purified from plasma using affinity chromatography, such as lysine-sepharose. In cell culture, serum is a source of plasminogen.[45] Following a single proteolytic cleavage event by one of its activators, plasminogen becomes active plasmin, a two-chain protein linked by a single disulfide bond.[37]

Plasminogen activators

Plasminogen activators, tPA and uPA, are serine proteases named for their sources at the time of their identification, tissue and urine. The equivalence of phenotypes of homozygous plasminogen knock-out mice and homozygous double knock-outs for the two activators suggests that there are no other plasminogen activators.[38] Both activate plasminogen by cleavage of its Arg561-Val562 bond; plasminogen is the only natural substrate identified so far for tPA and uPA, but many synthetic Lys or Arg substrates have been identified.[36] They are considered to be the key regulators of connective tissue remodeling.[39]

tPA

Tissue-type plasminogen activator (tPA, 72 kDa, 70 pM circulating concentration) regulates physiological fibrinolysis. It contains five domains: an amino-terminal fibronectin-like finger domain, putatively involved in fibrin binding; a growth factor-like domain with which it binds to its receptors; two kringle domains, the second of which is implicated in fibrin binding; and a carboxy-terminal serine protease domain. tPA lacks an amino-terminal pro-activation peptide as is found in plasminogen and uPA. Instead, tPA is active in its single chain form. It is primarily synthesized by endothelial cells but also by monocytes and other hematopoietic cells. In circulation, most tPA is bound to its primary inhibitor, PAI-1, as a complex that is removed from circulation by the liver. Otherwise it is found receptor-bound to endothelial cells or hepatocytes or bound to fibrin. tPA binds to fibrin with a dissociation constant on the order of 500 nM. In the absence of fibrin, tPA does not efficiently activate plasminogen, and a complex of the three, in which the dissociation constant for tPA is 20 nM, is required for efficient activation. tPA can activate Glu-plasminogen bound to an internal lysine of fibrin, a lysine that does not require fibrin degradation for exposure, and this activation is important for the initiation of fibrinolysis.[24, 37]

uPA

In tissues, plasminogen is activated to plasmin by urokinase (uPA, 54 kDa, 40 pM circulating concentration). uPA is found in large quantities in connective tissues. It is secreted by fibroblasts and most other cell types as a zymogen, pro-uPA, single chain polypeptide. The zymogen pro-uPA is activated by the same mechanism that activates plasminogen. Plasmin, as well as other enzymes that cleave pro-uPA at Lys158,

proteolytically converts pro-uPA to an active two-chain disulfide-bonded protein. uPA contains a growth factor-like domain that interacts with the uPA receptor, a kringle domain which lacks fibrin affinity, and a serine protease domain. Unlike tPA, uPA requires partial degradation of fibrin and exposure of zwitterionic lysines in order to activate Glu-plasminogen. While tPA is primarily active in maintaining vessel patency through its direct interaction with fibrin, uPA is active in tissue remodeling mediated by pericellular proteolysis and for which the binding of uPA to its cellular receptor is important.[37, 40]

Plasminogen activator receptors

uPA and tPA bind by their epidermal growth factor-like domains to their receptors. A 40 kDa receptor for tPA has been identified on the surface of endothelial cells. The receptor also binds plasminogen at affinities similar to the binding of plasminogen to the entire cell surface. tPA and plasminogen occupy two distinct binding domains of the receptor which catalyzes pericellular plasmin activation. A separate class of receptor, found on hepatocytes, mediates clearance of tPA from circulation. And tPA complexes with its primary inhibitor, PAI-1, are cleared by binding serpin receptors on hepatocytes.[41]

The receptor for urokinase (u-PAR, 48-60 kDa) is distinct from that of tPA and is found on a variety of cell types that produce pro-uPA. pro-uPA rapidly saturates cell surface binding sites in an autocrine fashion. The uPAR binds pro-uPA in such a way that it can be activated at the cell surface by plasmin generated nearby, leading to a rapid acceleration in the rate of plasmin activation. Rates of plasmin activation are also increased by binding of plasminogen to the cell-surface in the vicinity of the uPA-uPAR complexes.[40] On migrating cells, uPARs are found at the leading edge of the cells.[42] While it lacks its own intracellular

signaling activity, uPAR interacts with integrins in the cell membrane. These interactions mediate signaling cascades which alter cell adhesion, proliferation, and migration.[43]

Plasmin

Plasmin (≈ 85 kDa) is a serine protease. It is the primary enzyme responsible for resolution of hemostasis and maintenance of vascular patency.[44] In that role, plasmin degrades the clotting factors V, VIII, and XIIa, as well as fibrin. Plasmin initiates wound healing into sites of tissue injury and is both directly and indirectly involved in degradation of extracellular matrix. Plasmin itself degrades matrix molecules, such as fibronectin, laminin, type IV collagen, and proteoglycan core protins, and plasmin activates other degradative enzymes, predominantly MMPs. Finally, plasmin releases sequestered growth factors, such as FGFs and TGF-β from ECM, and TGF-β participates in feedback regulation of plasmin activation, upregulating PAIs and MMP inhibitors (TIMPs).[45]

Plasmin plays a major role in many physiological as well as pathological processes. It is important in development, tissue repair, neuronal plasticity,[46] inflammation,[47] and angiogenesis.[48] When misregulated, it is also involved in post-surgical adhesions,[49] tumor invasion[50] and metastasis,[51] destructive joint diseases, such as osteoarthritis[52] and rheumatoid arthritis,[53] and potentially also in brain damage following stroke or other trauma which compromises the blood-brain barrier.[54] The plasminogen activation system has also been appropriated by some strains of invasive human pathogens.[55]

Inhibitors of fibrinolysis

Protease inhibitors are a large protein fraction of human plasma. Most of them inhibit the serine proteases that initiate and carry out coagulation and fibrinolysis. Inhibition of serine proteases is typically governed by the formation of tightly bound complexes as opposed to transient ones formed by competing substrates that hydrolyze and disassociate more quickly. In cases where inhibitor hydrolysis does occur, there has been an evolution such that the inhibitor active site is not hydrolyzed and the inhibitor is not fully released from the protease. Inhibitor active sites typically contain a loop of approximately eight amino acid residues maintained by disulfide bonds at the base of the loop and are presented by the host inhibitor protein in a relatively rigid conformation.[56]

Serpins, PAIs

Serpins (**ser**ine **p**rotease **in**hibitors) are a family of proteins, many, but not all, of which are inhibitors of serine proteases. The exact mechanism of the protease inhibitors in the family is not known precisely due to incomplete crystal structures and limited mechanistic data. The serpins have an Arg residue at their reactive center. The serpin proteins alone are conformationally unstable and form stable equimolar complexes with their target proteases. The serpins include ATIII, PAI-1 PAI-2, PAI -3, and protease Nexin.[56]

Plasminogen activator-1 (PAI-1, 52 kDa) is the primary circulating inhibitor of tPA and two chain uPA. Its gene expression is highly regulated by hormones, cytokines, and growth factors and is upregulated by inflammation. PAI-1 is produced by endothelial cells and stimulated hepatocytes, predominantly, as well as smooth muscle cells. It circulates at a concentration of 10 μg/L or 200 pM where it forms a stoichiometric complex with

plasminogen activators; the complex is reversible, with a half-life of 90 min. However, complexes bind rapidly to α2-macroglobulin/lipoprotein-receptor-related proteins which are endocytic receptors.[34] In the plasma of healthy individuals, PAI-1 is always in molar excess over tPA. Nevertheless, due to the relatively slow synthesis of PAI-1 and inactivation of t-PA by PAI-1, there is usually a small quantity of t-PA in the active form, which is important for initiation of fibrinolysis.[37]

PAI-2 (70 kDa, < 70 pM) inhibits two chain uPA more rapidly than tPA. Circulating concentrations in healthy individuals are essentially unmeasurable, and therefore PAI-2 is not thought to play a role in the regulation of intravascular clot lysis but rather in controlled proteolyis in tissues.[37]

α2-plasmin inhibitor

α2-plasmin inhibitor (antiplasmin, 70 kDa) is the primary inhibitor of plasmin activity in the blood. It is a single chain protein synthesized in the liver and circulating at approximately 70 mg/L (1 μM), approximately half that of plasminogen on a molar basis. It is rapidly cross-linked to fibrin α chains during clot formation.[37] α2-plasmin inhibitor is a rapid and very high affinity inhibitor of plasmin in solution; it can also form a complex with plasmin when it is bound to fibrin and can block plasminogen binding to fibrin. The activity of α2-plasmin inhibitor is mediated by its many lysine residues at its carboxy-terminus, which bind Lys-binding sites in plasmin(ogen) kringle domains. Additionally, α2-plasmin inhibitor presents its Arg354-Met355 site as a competitive inhibitor for plasmin; following hydrolysis, the carboxy-terminal portion of α2-plasmin inhibitor remains bound to plasmin's

lysine binding sites. The presence and activity of α2-plasmin inhibitor are critical to controlling rates of clot dissolution, and its deficiency leads to hemorrhagic disorders.[29]

α2-macroglobulin

α2-macroglobulin (725 kDa) is a homotetrameric protein and a broad-specificity protease inhibitor circulating at 2.5 g/L (3 μM). It serves as a fail-safe mechanism for protease inhibition, for example when excessive plasmin is produced and α2-plasmin inhibitor quantities are insufficient. α2-macroglobulin has two reactive sites. First it has a bait region, Arg681ValGlyPheTyrGlu686. Any bond in the region can be cleaved depending on the protease. Each cleavage induces a conformational change in α2-macroglobulin, exposes a hydrolytically unstable thioester, and leads to covalent binding of Glu952 to the protease.[37]

MMPs

Matrix metalloproteinases (MMPs) are the second major class of enzymes responsible for breakdown of ECM and are unique in their ability to degrade collagens. They are produced in a variety of cell types, including fibroblasts, macrophages, neutrophils, and keratinocytes, as well as bacteria and molds. MMPs require divalent cations, typically Ca++ or Zn++, for activity. As many as twenty-two MMPs have been identified. They cleave specific types of collagen at specific sites. For a number of MMPs, enzyme substrate kinetics have been well described.[57] MMPs are carefully regulated; they are maintained under transcriptional control, are secreted in a latent form that is activated by cleavage of a

propeptide, and are inhibited both by specific tissue inhibitors of MMPs (TIMPs) and by α2-macroglobulin which circulates at relatively high concentrations.[45]

Cell migration

For a cell to move in on a two-dimensional substrate it must adhere to the substrate. For a cell to migrate in a three-dimensional environment, e.g., most physiological environments, it must also degrade extracellular matrix. Cells must also degrade matrices in order to remodel them, e.g., to lay down a new substrate, and in order to proliferate. In tissues and at tissue barriers, matrix degradation is primarily performed by proteolytic enzymes in order for migrating cells to penetrate. In normal physiological situations, migration or invasion of cells is limited, and tissue destruction and remodeling should be limited in extent and duration and therefore must be regulated carefully to start, stop, and localize the proteolysis. Therefore proteolysis is pericellular. In a tissue injury, a provisional fibrin matrix is formed. To penetrate plasma clots, cells generate local zones of fibrinolysis.[58, 59, 60] Remodeling of the fibrin is performed progressively by cells such as macrophages and fibroblasts which infiltrate the matrix from the wound margins. Infiltrating cells histochemically stain positively for plasminogen activators and plasminogen activator receptors, and plasminogen is dispersed throughout the wound bed.[42]

SYNTHETIC BIOMATERIALS

To avoid long-term inflammation and other adverse immune responses to implants and to provide a mechanism for the most biological and integrated tissue repair possible, degradable synthetic biomaterials are desirable and are already common in clinical use.

Degradable synthetic sutures are the prototypical example. They have been commercially available since the 1970s[61] and most are made from a family of polyesters, poly (α-hyroxy acids), which include poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and their co-polymers, poly(lactic-co-glycolic acid) (PLGA). Poly(α-hydroxy acids) are FDA approved for certain applications. They can be readily processed into a variety of shapes and forms, including macroporous scaffolds for tissue engineering.[62] They, like most synthetic biodegradable polymers, degrade by passive hydrolysis.[63] The degradation products of PLA, PGA, and PLGA are natural metabolites; however, they are acidic and they lower the pH in the local environment of the implant, leading to autocatalytic material degradation and potentially causing reduced cell viability. Also, the poly(α-hydroxy acids) must be modified to render them sufficiently cell-adhesive.[64]

The use of absorbable polymeric materials for orthopedic applications has been reviewed by An et al. Briefly, poly(α-hyroxy esters) such as poly(lactic acid), poly(glycolic acid), and their copolymers, PLGA foams and fiber meshes, are being broadly evaluated (e.g., optimal pore sizes are approximately 250 μm[65]). However, they undergo bulk degradation with rapid loss of mechanical properties, must be surface treated for cell attachment, and elicit an inflammatory tissue response, presumably due to crystalline degradation products.[66] A class of tyrosine-derived polycarbonates are also based on natural metabolites but do not degrade into crystalline particles like the poly(α-hyroxy esters) because they are amorphous and do not degrade into acidic products like the poly(α-hyroxy esters).[67] Polypropylene fumarates,[63] functionalized polyphosphazenes with backbones of alternating nitrogen and phosphorous, and polyanhydrides are also being evaluated for potential osteoconductivity and biocompatibility. Compared to naturally derived

biomaterials, these synthetic materials reduce the risks of immunogenicity and disease transmission, and by being degradable they should avoid the production of wear particles that result from nondegradable polymeric implants. Their supplies are generally not limited and syntheses can be tailored to meet clinical needs once they are established.

New degradable synthetic materials are being developed to overcome some of the weaknesses of poly(α-hydroxy acids). Tyrosine-derived polycarbonates of desaminotyrosyl-tyrosine ethyl ester and desaminotyrosyl-tyrosine hexyl ester are examples.[67, 68] Their mechanical properties are comparable with poly(α-hydroxy acids) and their degradation products are more pH neutral. However, their degradation rates are slow, on the order of months to years, which may hinder tissue repair processes that should displace an implant.

Slow degradation rates can be desirable for long gap nerve growth guides, for example. Strong, flexible poly(trimethylene carbonate-*co*-ε-caprolactones) are being developed for such applications. With greater than 30% trimethylene carbonate content, the polymers are amorphous, have low degrees of swelling, and can be drawn into fibers and wound into desired geometries, such as tubes.[69]

Another category of degradable synthetic biomaterials are polyesterurethanes (DegraPol) formed by polyaddition of two macrodiols.[70] Due to alternating, phase-separated blocks of crystalline poly(hydroxybutyric acid] and amorphous poly(ε-caprolactone), the materials are elastomeric and can have good mechanical properties (elastic modulus 30 MPa – 1.2 GPa), and degradation times from weeks to years. In addition to hydrolytic degradation, however, phagocytosis by macrophages and osteoblasts was also observed *in vivo*.

However, what all of these synthetic degradable materials lack is sufficient biomimetic character. They cannot be formed *in situ* to match tissue architecture or to use minimally invasive surgical techniques. And, while they degrade by passive hydrolysis, they are not cell-responsive. The timing of their degradation follows an essentially pre-programmed chemical clock. Degradation rates are affected by water uptake (due to the porosity and hyrophobicity), crystallinity, bond type and bond microenvironment.[71]

IN SITU MATERIAL FOMATION

In situ means "in the natural position or original location" or simply "in place." To form a biomaterial *in situ* therefore means to make it in its intended site of application, which, for an implant, means to make it in contact with tissues and cells. Naturally occurring biomaterials such as fibrin and ECM are of course formed *in situ*. Such material formations take place gently by shifting molecules from solution phase to solid phase using mechanisms such as self-assembly, precipitation, and enzymatic cross-linking. There are many advantages to making a material *in situ*. The resulting material obtains exact shape matching to the underlying or surrounding tissue, which can have quite complex architecture. By delivering a liquid that will be transformed to a solid after application, one has the capability to deliver a potentially large volume material through a relatively small hole in the body using minimally invasive surgical techniques.[72] However, one is also subject to a number of constraints when forming a material *in situ*. The material formation time should be relatively rapid, a few seconds or minutes, depending on the type of surgery. It is desirous to avoid organic solvents whenever possible to avoid toxicity and denaturation of biological molecules such as proteins; therefore neat or hydrophilic precursors in aqueous solutions are

desirable. The phase change should occur at body temperature and should not require a significant endo- or exothermic process. Also, the precursors, their reaction products, and their degradation products should not be toxic nor react significantly with molecules in the tissue environment.

Such gentle transformations can be achieved by a number of means: precipitation;[73] self-assembly of peptides by electrostatic interactions;[74] physical cross-linking of polymers in sudden response to a change in an environmental condition such as temperature or pH;[75, 76, 77, 78, 79] thermoforming;[73] photocross-linking;[80] photopolymerization;[81, 82] and enzymatic cross-linking.[83] In the first three examples, materials are formed by physical-chemical associations and without covalent cross-linking. In these cases, the materials tend to suffer from poor mechanical properties and short lifetimes due to relatively rapid reversibility of the formation process. To form more stable materials, covalent cross-linking is often required.[84]

As a technique for *in situ* biomaterial formation, covalent cross-linking usually fails due to the toxicity of low molecular weight initiators or monomers and due to tissue-damaging heats of reaction. However, biomaterials can be formed from mixtures of reactive macromolecular precursors, e.g., by condensation of a polymer that contains many amines with a polymer that contains many amine-reactive functionalities, such as N-hydroxysuccinimidyl active esters.[85] However, amines on molecules in the extracellular space where the biomaterial is formed are also reactive with N-hydroxysuccinimiyl esters. Other reactive schemes to forming biomaterials *in situ* should be possible by choosing precursors selective for each other relative to molecules in the extracellular environment. There are many medical applications for *in situ* forming materials: localized drug delivery, e.g., of an anti-cancer agent to a tumor site; depot delivery of a drug for sustained release,

e.g., human growth hormone; prevention of thrombosis and intimal thickening after balloon angioplasty;[86] prevention of adhesions, e.g. postoperative adhesions in gynecological procedures;[87] immunoisolation for cell transplantation, e.g., as treatment of diabetes;[88] as a sealant, e.g., in conjunction with or in lieu of sutures; and as an injectable scaffold for wound healing and tissue repair, e.g., to bony non-unions. Drug delivery applications have been reviewed by West[84] and are of particular interest for delivery of peptide and protein drugs due to their ineffectiveness following oral delivery.

OBJECTIVES OF THIS THESIS

The goal of this work was the design, synthesis, and characterization of a novel class of biomaterials using completely synthetic precursors. The materials were to be formed *in situ* and to be degraded on demand by cells themselves. Developing a method with which to demonstrate the degradability of the materials by cells was not trivial and became a significant goal in and of itself. The ultimate goal of the work was to demonstrate healing responsiveness of the materials *in vivo*.

There are many reasons to form a biomaterial *in situ*, e.g., at the site of implantation, and a good way to do this is to induce a liquid-solid transformation relatively rapidly following material application. Handling liquids and syringes is something that surgeons, the intended users of biomaterials, are accustomed to doing. Delivering a liquid through a small hole is a relatively non-invasive way to deliver a potentially large volume material, thus reducing excessive trauma due to a large incision or to manipulation of tissues and organs. Allowing a liquid-to-solid transition to occur *in situ* gives shape matching to tissue and defect architecture which is rarely possible with preformed materials.

Degradable biomaterials are also desirable for many reasons. They may be used to deliver drugs locally rather than systemically or continuously over long periods of time in order to avoid frequent painful injections. They may serve as scaffolds for tissue repair where a space-filling object is required until the body has replaced it, a temporary foothold for cells, by its own extracellular matrix. And the use of degradable materials should reduce or prevent long term inflammatory and immune responses often affiliated with permanent implants. Materials that degrade in response to cellular activity are even more desirable since tissue repair or healing is a cell-mediated process. Its rate depends on the tissue type, extent and type of disease or injury, and individual patient.

The materials described herein satisfy the criteria above. They can be formed *in situ* using water soluble precursors and selective conjugate addition reactions. With a novel three-dimensional cell migration assay, the materials were demonstrated to possess at least the minimum biological information necessary for cell adhesion and migration, the latter mediated by plasmin, an endogenous mechanism for cell migration. In addition, the materials are completely synthetic, thus avoiding the problems associated with the use of human- and other animal-derived materials. And finally, they can be used to deliver active growth factors and can induce tissue morphogenesis *de novo* as demonstrated in a calvarial critical size defect model.

REFERENCES

[1] Langer R & Vacanti JP. Tissue Engineering. *Science* **260**, 920-926 (1993).

[2] Rouhi AM. Biomaterials body shop: diverse approaches to diverse human needs. *Chemical & Engineering News*. **78**, 33-41 (2000).

[3] Colman RW, Marder VJ, Salzman EW & Hirsh J. Overview of hemostasis. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice, Third Edition.* Colman RW, Hirsh J, Marder VJ & Salzman EW (eds). (J.B. Lippincott Company, Philadelphia, PA, USA, 1994).

[4] Altieri DC, Plescia J, Plow EF. The structural motif glycine 190-valine 202 of the fibrinogen gamma chain interacts with CD11b/CD18 integrin (alpha M beta 2, Mac-1) and promotes leukocyte adhesion. *J. Biol. Chem.* **268**, 1847-1853 (1993).

[5] Andrieux A et al. Amino acid sequences in fibrinogen mediating its interaction with its platelet receptor, GPIIbIIIa. *J. Biol. Chem.* **264**, 9258-9265 (1989).

[6] Gartner TK & Bennett JS. The tetrapeptide analogue of the cell attachment site of fibronectin inhibits platelet aggregation and fibrinogen binding to activated platelets. *J. Biol. Chem.* **260**, 11891-11894 (1985).

[7] Smith JW, Ruggeri ZM, Kunicki TJ, Cheresh DA. Interaction of integrins alpha v beta 3 and glycoprotein IIb-IIIa with fibrinogen: differential peptide recognition accounts for distinct binding sites. *J. Biol. Chem.* **265**, 12267-11271 (1990).

[8] Doolittle R, Goldbaum D, Doolittle L. Designation of sequences involved in the "coiled coil" interdomainal connector in fibrinogen: construction of an atomic scale model. *J. Mol. Biol.* **120**, 311 (1978).

[9] Kolev K, Komorowicz E, Owen WG & Machovich R. Quantitative comparison of fibrin degradation with plasmin, miniplasmin, neutrophil leukocyte elastase and cathepsin G. *Thrombosis and Haemostasis* **75**, 140-6 (1996).

[10] Mintz PD et al. Fibrin sealant: clinical use and the development of the University of Virginia Tissue Adhesive Center. *Annal. Clin. Lab. Sci.* **31**, 108-117 (2001).

[11] Dunn CJ & Goa KL. Fibrin sealant: a review of its use in surgery and endoscopy. *Drugs* **58**, 863-886 (1999).

[12] Deutsch M, Meinhart J, Fischlein T, Preiss P & Zilla P. Clinical autologous in vitro endothelialization of infrainguinal ePTFE grafts in 100 patients: a 9-year experience. *Surgery* **126**, 847-855 (1999).

[13] Schense JC, Bloch J, Aebischer P & Hubbell JA. Enzymatic incorporation of bioactive peptides into fibrin matrices enhances neurite extension. *Nature Biotechnol.* **18**, 415-419 (2000).

[14] Sakiyama-Elbert SE & Hubbell JA. Development of fibrin derivatives for controlled release of heparin-binding growth factors. *J. Control. Rel.* **65**, 389-402 (2000).

[15] Sakiyama-Elbert SE, Panitch A & Hubbell JA. Development of growth factor fusion proteins for cell-triggered drug delivery. *FASEB J.* (published online March 20, 2001).

[16] Hay ED (ed.). *Cell Biology of Extracellular Matrix, Second Edition.* (Plenum Press, New York, 1991).

[17] Streuli CH & Grant ME (eds). *Methods in Molecular Biology **139** Extracellular Matrix Protocols* (Humana Press Inc., Totowa, NJ, USA, 2000).

[18] Friess W. Collagen – biomaterial for drug delivery. *Eur. J. Pharm. Biopharm.* **45**, 113-136 (1998).

[19] Linsenmayer TF. Collagen. *Cell Biology of Extracellular Matrix, Second Edition.* Hay ED (ed.). (Plenum Press, New York, 1991).

[20] Neldner KH. Pseudoxanthoma elasticum. *Connective Tissue and Its Heritable Disorders: Molecular, Genetic, and Medical Aspects.* Royce PM & Steinmann B (eds). (Wiley-Liss, Inc., New York, 1993).

[21] Kuijpers AJ et al. Cross-linking and characterization of gelatin matrices for biomedical applications. *J. Biomater. Sci. Polymer Edn.* **11**, 225-243 (2000).

[22] Rao KP. Recent developments of collagen-based materials for medical applications and drug delivery systems *J. Biomater. Sci. Polym. Edn.* **7**, 623-645 (1995).

[23] Mecham RP & Heuser JE. The Elastic Fiber. *Cell Biology of Extracellular Matrix, Second Edition.* Hay ED (ed.). (Plenum Press, New York, 1991).

[24] Hantgan RR, Francis CW & Marder VJ. Fibrinogen structure and physiology. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice, Third Edition.* Colman RW, Hirsh J, Marder VJ & Salzman EW (eds). (J.B. Lippincott Company, Philadelphia, 1994).

[25] Yamada KM. Fibronectin and other cell interactive glycoproteins. *Cell Biology of Extracellular Matrix, Second Edition.* Hay ED (ed.). (Plenum Press, New York, 1991).

[26] Kleinman HK et al. The laminins: a family of basement membrane glycoproteins important in cell differentiation and tumor metastases. *Vitamins Hormones* **47**, 161-186 (1993).

[27] Hall, H & Schachner, M. Laminins and their ligands: involvement of carbohydrates in formation of the extracellular matrix and in cell adhesion. *Trends in Glycoscience and Glycotechnology* **10**, 361-382 (1998).

[28] Wight TN, Heinegård DK & Hascall VC. Proteoglycans: structure and function. *Cell Biology of Extracellular Matrix, Second Edition.* Hay ED (ed.). (Plenum Press, New York, 1991).

[29] Colman RW, Marder VJ, Salzman EW & Hirsh J. Overview of hemostasis. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice, Third Edition.* Colman RW, Hirsh J, Marder VJ & Salzman EW (eds). (J.B. Lippincott Company, Philadelphia, 1994).

[30] Pierschbacher MD & Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* **309**, 30-33 (1984).

[31] Yamada Y & Kleinman HK. Functional domains of cell adhesion molecules. *Curr. Opin. Cell Biol.* **4**, 819-823 (1992).

[32] Hubbell JA. Biomaterials in tissue engineering. *Biotechnology* **13**, 565-576 (1995).

[33] Felsenfeld DP, Choquet D & Sheetz MP. Ligand binding regulates the directed movement of β1 integrins on fibroblasts. *Nature* **383**, 438-440 (1996).

[34] Chapman HA. Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration. *Curr. Opin. Cell Biol.* **9**, 714-724 (1997).

[35] Murhpy G & Reynolds JJ. Extracellular matrix degradation. *Connective Tissue and Its Heritable Disorders: Molecular, Genetic, and Medical Aspects.* Royce PM & Steinmann B (eds). (Wiley-Liss, Inc., New York, 1993).

[36] Kiel B. *Specificity of proteolysis.* (Springer-Verlag, New York, 1992).

[37] Bachmann F. The plasminogen-plasmin enzyme system. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice, Third Edition.* Colman RW, Hirsh J, Marder VJ & Salzman EW (eds). (J.B. Lippincott Company, Philadelphia, 1994).

[38] Ploplis VA et al. Effects of disruption of the plasminogen gene on thrombosis, growth, and health in mice. *Circulation* **92**, 2585-2593 (1995).

[39] Plow EF, Herrn T, Redlitz A, Miles LA & Hoover-Plow JL. The cell biology of the plasminogen system. *FASEB J.* **9**, 939-945 (1995).

[40] Ellis et al. *Plasminogen activation in fibrinolysis, in tissue remodeling, and in development* **667** Brakman P & Kluft C (eds). (Annals of the New York Academy of Science, New York, 1992).

[41] Hajjar KA & Nachman RL. The human endothelial cell plasmin-generating system. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice, Third Edition.* Colman

RW, Hirsh J, Marder VJ & Salzman EW (eds). (J.B. Lippincott Company, Philadelphia, 1994).

[42] Schäfer BM, Maier K, Eickhoff U, Todd RF & Kramer MD. Plasminogen activation in healing human wounds. *Amer. J. Pathol.* **144**, 1269-1280 (1994).

[43] Ossowski L & Aguirre-Ghiso JA. Urokinase receptor and integrin partnership: coordination of signaling for cell adhesion, migration and growth. *Curr. Opin. Cell Biol.* **12**, 613-620 (2000).

[44] Plow EF, Herren T, Redlitz A, Miles LA & Hoover-Plow JL. The cell biology of the plasminogen system. *FASEB J.* **9**, 939-945 (1995).

[45] Murphy G, Atkinson S, Ward R, Gavrilovic J & Reynolds JJ. The role of plasminogen activators in the regulation of connective tissue metalloproteinases. *Plasminogen activation in fibrinolysis, in tissue remodeling, and in development* **667** Brakman P & Kluft C (eds). (Annals of the New York Academy of Science, New York, 1992).

[46] Seeds NW et al. Plasminogen activators and their interaction with the extracellular matrix in neural development, plasticity and regeneration. *Semin. Neurosci.* **8**, 405-412 (1996).

[47] Heiden M, Seitz R & Egbring R. The role of inflammatory cells and their proteases in extravascular fibrinolysis. *Semin. Thromb. Hemost.* **22**, 497-501 (1996).

[48] Pintucci G, Bikfalvi A, Klein S & Rifkin DB. Angiogenesis and the fibrinolytic system. *Semin. Thromb. Hemost.* **22**, 517-524 (1996).

[49] Holmdahl L. The role of fibrinolysis in adhesion formation. *Eur. J. Surg. Suppl.* **577**, 24-31 (1997).

[50] Thorgeirsson UP, Lindsay CK, Cottam DW & Gomez DE. Tumor invasion, proteolysis, and angiogenesis. *J. Neuro-oncology* **18**, 89-103 (1994).

[51] Andreasen PA, Kjoller L, Christensen L & Duffy MJ. The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int. J. Cancer* **72**, 1-22 (1997).

[52] Serni I et al. Plasminogen activator and receptor in osteoarthritis. *J. Rheumatol.* **22**, 120-122 (1995).

[53] Del Rosso M, Fibbi G & Matucci Cerinic M. The urokinase-type plasminogen activator system and inflammatory joint diseases. *Clin. Exp. Rheumatol.* **17**, 485-498 (1999).

[54] Gingrich MB & Traynelis SF. Serine proteases and brain damage: is there a link? *Trends Neurosci.* **23**, 399-407 (2000).

[55] Boyle MDP & Lottenberg R. Plasminogen activation by invasive human pathogens. *Thrombosis & Haemostasis* **77**, 1-10 (1997).

[56] Salvesen G & Pizzo SV. Proteinase inhibitors: α-macroglobulins, serpins, and kunins. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice, Third Edition*. Colman RW, Hirsh J, Marder VJ & Salzman EW (eds). (J.B. Lippincott Company, Philadelphia, 1994).

[57] Netzel-Arnett S, Fields G, Birkedal-Hansen H & Van Wart HE. Sequence specificities of human fibroblast and neutrophil collagenases. *J. Biol. Chem.* **266**, 6747-6755 (1991).

[58] Saksela O. Plasminogen activation and regulation of pericellular proteolysis. *Biochim. Biophys. Acta* **823**, 35-65 (1985).

[59] Knox P, Crooks S, Scaife MC & Patel S. Role of plasminogen, plasmin, and plasminogen activators in the migration of fibroblasts into plasma clots. *J. Cell. Physiol.* **132**, 501-508 (1987).

[60] Anglés-Cano E. Overview of fibrinolysis: plasminogen activation pathways on fibrin and cell surfaces. *Chem. Phys. Lipids.* **67/68**, 353-362 (1994).

[61] Jacoby M. Custom-made biomaterials. *Chemical & Engineering News* **79**, 30-35 (2001).

[62] Chaignaud BE, Langer RS & Vacanti JP. The history of tissue engineering using biodegradable synthetic polymer scaffolds and cells. *Synthetic Biodegradable Polymer Scaffolds*. Atala A & Mooney D (eds). (Birkhäuser, Boston, MA, USA, 1997).

[63] Behravesh E, Yasko AW, Engel PS & Mikos AG. Synthetic biodegradable polymers for orthopaedic applications. *Clinical Orthopaedics and Related Research* **367S**, S118-125 (Lippincott Williams & Wilkins, Inc. 1999).

[64] Berrera DA, Zylstra E, Lansbury PT & Langer R. Synthesis and RGD peptide modification of a new biodegradable copolymer system: poly(lactic acid-co-lysine. *J. Amer. Chem. Soc.* **115**, 11010-11011 (1993).

[65] Kenley R, Marden L, Turek T, Jin L, Ron E, & Hollinger JO. Osseos regeneration in the rat calvarium using novel delivery systems for recombinant human bone morphogenetic protein-2 (rhBMP-2). *J. Biomed. Mater. Res.* **28**, 1139-1147 (1994).

[66] Groeneveld EHJ & Burger EH. Bone morphogenetic proteins in human bone regeneration. *Eur. J. Endocrinology* **142**, 9-21 (2000).

[67] Choueka J et al. Canine bone response to tyrosine-derived polycarbonates and poly(L-lactic acid). *J. Biomed. Mat. Res.* **31**, 35-41 (1996).

[68] Ertel SI & Kohn J. Evaluation of a series of tyrosine-derived polycarbonates as degradable biomaterials. *J. Biomed. Mater. Res.* **28**, 919-930 (1994).

[69] Pêgo AP, Grijpma DW, Poot AA & Feijen J. Poly(ester carbonates) for artificial nerve guides. *Proceedings of the European Society for Biomaterials* (Bordeaux-Arcachon, France, September 8-12, 1999).

[70] Saad B et al. Development of degradable polyesterurethanes for medical applications: *in vitro* and *in vivo* evaluations. *J. Biomed. Mater. Res.* **36**, 65-74 (1997).

[71] Hubbell JA. Bioactive biomaterials. *Curr. Opin. Biotechnology* **10**, 123-129 (1999).

[72] Hubbell JA. *In situ* material transformations in tissue engineering. *MRS Bulletin* **21**, 33-35 (1996).

[73] Slepian MJ. Polymeric endoluminal paving. *Cardiol. Clin.* **12**, 715-737 (1994).

[74] Holmes TC et al. Extensive neurite outgrowth and active synapse formation on self-assembling peptide scaffolds. *Proc. Nat. Acad. Sci.* **97**, 6728-6733 (2000).

[75] Chen GH & Hoffman AS. Graft-copolymers that exhibit temperature-induced phase-transitions over a wide range of pH. *Nature* **373**, 49-52 (1995).

[76] Yoshida R, et al. Comb-type grafted hydrogels with rapid de-swelling response to temperature changes. *Nature* **374**, 240-242 (1995).

[77] Jeong B, Bae YH, Lee DS & Kim SW. Biodegradable block copolymers as injectable drug-delivery systems. *Nature* **388**, 860-862 (1997).

[78] Petka WA, Harden JL, McGrath KP, Wirtz D & Tirrell DA. Reversible hydrogels from self-assembling artificial proteins. *Science* **281**, 389-392 (1998).

[79] Galaev IY & Mattiasson B. 'Smart' polymers and what they could do in biotechnology and medicine. *Trends in Biotechnology* **17**, 1999, 335-340.

[80] Moghaddam MJ & Matsuda T. Molecular design of three-dimensional artificial extracellular matrix: photosensitive polymers containing cell adhesive peptide. *J. Polym. Sci. A Polym. Chem.* **31**, 1589-1597 (1993).

[81] Pathak CP, Sawhney AS & Hubbell JA. Rapid photopolymerization of immunoprotective gels in contact with cells and tissues. *JACS* **114**, 8311-8312 (1992).

[82] Sawhney AS, Pathak CP & Hubbell JA. Bioerodible hydrogels based on photopolymerized poly(ethylene glycol)-*co*-poly(*a*-hydroxy acid) diacrylate macromers. *Macromolecules* **26**, 581-587 (1993).

[83] Sperinde JJ & Griffith LG. Synthesis and characterization of enzymatically-cross-linked poly(ethylene glycol) hydrogels. *Macromolecules* **30**, 5255-5264 (1997).

[84] West JL. *In situ* formation of polymer matrices for localized drug delivery. *Therapeutic Protein and Peptide Formulation and Delivery* **675**, 119-123 (1997).

[85] Morpurgo M, Veronese FM, Kachensky D & Harris JM. Preparation and characterization of poly(ethylene glycol) vinyl sulfone. *Bioconjug. Chem.* **7**, 363-368 (1996).

[86] West JL & Hubbell JA. Separation of the arterial wall from blood contact using hydrogel barriers reduces intimal thickening after balloon injury in the rat: the roles of medial and luminal factors in arterial healing. *Proc. Natl. Acad. Sci. USA* **93**, 13188-13193 (1996).

[87] Sawhney AS, Pathak CP, van Rensburg JJ, Dunn RC & Hubbell JA. Optimization of photopolymerized bioerodible hydrogel properties for adhesion prevention. *J. Biomed. Mater. Res.* **28**, 831-838 (1994).

[88] Cruise GM et al. In vitro and in vivo performance of porcine islets encapsulated in interfacially photopolymerized poly(ethylene glycol) diacrylate membranes. *Cell Transplantation* **8**, 293-306 (1999).

CHAPTER 2

MATERIAL DESIGN AND SYNTHESIS

Materials were designed to be formed by a chemical route well known among chemists but so far not exploited by biomaterials scientists. That chemistry is a Michael-type conjugate addition between a nucleophile, such as the thiolate, and a conjugated unsaturated functional group, such as the acrylate or vinylsulfone. The vinylsulfone was chosen for its known selectivity for thiols over other potentially competing nucleophiles found in biological environments. Similar specificity was demonstrated for the acrylate. Qualitative kinetic experiments in aqueous buffer at physiologic pH 7.4 were performed in order to confirm the selectivity of a PEG-monoacrylate for N-acetyl-cysteine relative to α-N-acetyl-lysine (ε-amine) and glycyl-glycine (α-amine). Also, the rate of disulfide bond formation of the N-acetyl-cysteine was shown to be considerably slower than the conjugate addition reaction. Rheometric techniques were used to study the kinetics of gel formation using the combination of a PEG-triacrylate and a PEG-dithiol or the combination of a PEG-divinylsulfone and a peptide containing three cysteines. The elastic (storage) moduli and rates of gel formation were studied, for example the latter as a function of pH over a range of interest for forming materials *in situ*. Also, sufficiently low toxicity of gel precursors at concentrations and exposure times relevant for gel formation was demonstrated in order to pursue material formation in contact with cells and tissues.

INTRODUCTION

It is desirable to have methods for forming biomaterials *in situ*, that is to say in direct contact with cells and tissues at the site of implantation or for implantation of live cells.[1] When one makes a material *in situ* one can obtain precise shape matching to sophisticated tissue architectures which is rarely possible with materials made *ex vivo* and then implanted (Figure 2. 1). Forming a material *in situ* also allows one to use minimally invasive surgical techniques to implant a relatively large material through a small hole. To do so, one applies a liquid precursor solution that undergoes a liquid-solid phase transformation shortly following delivery; the phase transformation may result from physical or chemical cross-linking or a phase change induced, for example, by a temperature change. To make a material *in situ*, one is constrained by biological conditions, which are wet and oxidizing, and by biological sensitivity to relatively small changes in temperature and pH and to even relatively low concentrations of most solvents other than water. Another general constraint on forming a material *in situ* is the time for the transformation to occur; the time must not be too short that a solid forms before proper delivery, and it must not be so long as to place a patient at risk, e.g., if patient circulation or breathing are suspended for a time in order to apply the biomaterial. And finally, any chemistry used to generate a biomaterial *in situ* should react as little as possible with cells and tissues in order to maintain cell viability and normal phenotype and genotype.

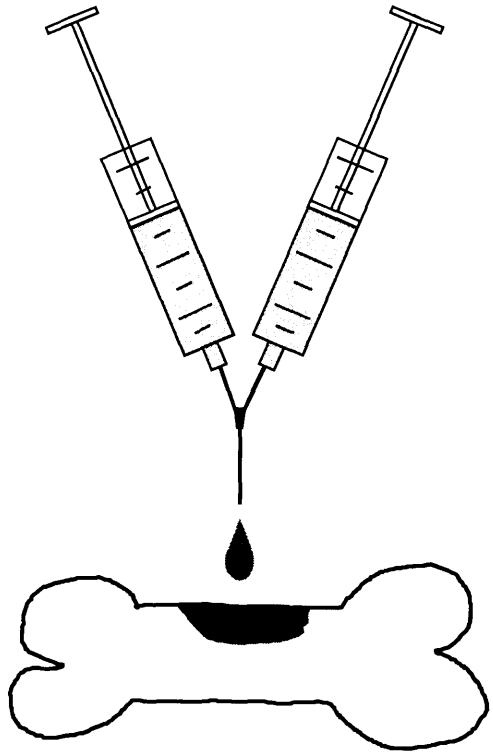

Figure 2. 1 Cartoon of *in situ* material formation

So far a number of relatively gentle material transformations have been described for synthetic biomaterial formation. These include precipitation;[2] thermoforming;[2] physical cross-linking of "smart" polymers in nonlinear response to external stimuli such as temperature or pH;[3, 4, 5, 6, 7] photocross-linking;[8] photopolymerization;[9] and enzymatic cross-linking (of PEG-derivatives with a polypeptide; transglutaminase cross-linking of glutamine derivatives of PEG with poly(Lys-Phe)).[10] Biomaterials can also be formed from mixtures of mutually chemically reactive macromolecular precursors, for example by condensation of a polymer that contains many amines with a polymer that contains multiple amine-reactive functionalities, such as N-hydroxysuccinimidyl esters.[11] However, such methods have the

disadvantage that they tend to have side reactions with functional groups on biological molecules on the cell surface and in the extracellular space, predominantly proteins.

Therefore, it is desirable to have self-selective reactions for making materials *in situ.* Self-selective means that the two or more reactants or material precursors react with each other much faster than with other molecules they contact during their reaction, for example proteins and carbohydrates in solution, on the cell surface, and in the extracellular matrix.

One published self-selective chemistry involves the thiol and vinylsulfone. Morpurgo et al.[12] used amino acid analysis, trinitrobenzenesulfonic acid, and dithiodipyridine assays to show that the reaction of PEG-monovinylsulfone (PEG-monoVS) with unreduced ribonuclease was relatively slow (100 hr at pH 9.3 and 21° C). At the same concentrations and temperature, reaction with reduced ribonuclease was shown to be relatively rapid (1 hr at pH 8.0) and selective at pH 7-9. PEG-monoVS did not modify any other amino acid residues besides cysteine (reduced protein) and lysine (reduced and unreduced protein). Reacting 10 fold excess PEG-monoVS with small molecules such as N-acetyl-cysteine and a-N-acetyl-lysine methyl ester (pH 8.0 and 21° C), the same researchers measured a half life of thiol reaction of 34 min compared to ≥ 24 hr for ε-amine reaction. Also, they observed that the covalent bond formed by reaction of N-acetyl-cysteine and PEG-monoVS was indefinitely stable in aqueous solution. The work of Morpurgo et al. was performed in the context of defining a general technique for synthesizing well-defined and stable PEG-grafted proteins. In this thesis, the same chemistry is described for the first time to make cross-linked biomaterials which are stable to passive hydrolysis but degradable by physiological mechanisms (Chapters 3-5).

For some applications, material degradation by passive hydrolysis is desirable.[13, 14] Delivery of active, unmodified therapeutic molecules (small molecule as well as macromolecular drugs such as proteins) in a controlled way is an important application of such degradable materials; the desired control may be in the dimension of time (e.g., long term delivery of a drug from a one time implant or injection) or in location (e.g., to a tumor with minimal systemic exposure). Techniques of *in situ* drug encapsulation for localized delivery and their limitations have been reviewed by West[15] and Hubbell.[16] Given the self-selectivity of the vinylsulfone and the sulfhydryl, it was hypothesized that a similar thiol specificity would be obtainable using an acrylate as the Michael-type acceptor and thus allow protein encapsulation without covalent protein modification. Networks made with acrylates would contain ester bonds between points of cross-linking, and such networks would be labile due to passive hyrolysis of the ester bonds at a rate determined by the environment of the ester bonds (e.g., slow hyrolysis and release in a hydrophobic material, fast in a highly water-swollen material).

Thiols are found in biological molecules, predominantly in cysteine-containing proteins and hence in affiliation with cells and tissues and therapeutic proteins. However, these thiols are not at significant risk of modification by Michael-type acceptors that may be used to form biomaterials *in situ*. There are two primary reasons for low rates of side reaction with thiols in biological systems: free cysteine is predominantly sequestered to the intracellular space, and the materials described herein are formed extracellularly where thiols are found predominantly in the disulfide-bonded cystine state.

The other potentially strong nucleophile present in extracellular biological molecules is the amine. Lysine residues of proteins provide an abundant source of amines in the

extracellular space; the ε-amines of lysine have pKa values of 9.2-10, depending on their microenvironment, and tend to be reactive above pH 8. When the pH is below 8, cysteine thiols, with pKa values of 7-8.5, will be more reactive as nucleophiles than the ε-amines of lysine. Amino-terminal amines of proteins have pKa values of 7-8, which are similar to pKa values of cysteine thiols. However, α-amines are found in relatively low concentrations, with one or fewer per polypeptide chain because many protein amino-termini are acetylated to reduce proteolytic degradation.[17, 18] Outside of the cell and the cell membrane, α,β-unsaturated carbonyls are found in few endogenous structures, e.g., some steroids, and hence in relatively low concentrations.

The acrylate or vinylsulfone groups were coupled to moderate molecular weight polymers of PEG in an effort to avoid the use of low molecular weight activators and cross-linkers and hence to reduce the toxicity of the starting materials. While vinylsulfone itself is genotoxic *in vitro*,[19] these effects come from the ability of vinylsulfone to penetrate the plasma and nuclear membranes and react with glutathione and DNA. When conjugated to PEG, an uncharged, hydrophilic macromolecule, the vinylsulfone is excluded from the cell by the impermeability of PEG through the cell membrane. The PEG makes the conjugated precursors highly water soluble as it is desirable to use aqueous precursor solutions when forming materials in contact with viable cells and tissues. PEG was also chosen for its biocompatibility, resistance to protein adsorption, and favorable toxicology.[20, 21]

MATERIALS AND METHODS

PEG-monoacrylate

PEG-monoacrylate was prepared from PEG-monomethylether, mol. wt. 5000 Da (Fluka, Buchs, Switzerland). PEG-monomethylether (Fluka) was dried by azeotropic distillation in toluene for 1 hour using a Dean-Stark trap. After cooling to less than 50 °C under argon, triethylamine (2 eq per PEG-OH) in dichloromethane was added. The reaction was started by drop-wise addition of acryloyl chloride (1.5 eq) The reaction proceeded with stirring overnight in the dark at room temperature under argon. The resulting pale yellow solution was filtered through a neutral alumina bed. Sodium carbonate was added to the toluene-dichloromethane solution and stirred for 2 hr. The sodium carbonate was removed by filtration, and the volume of the solution reduced by rotary evaporation. The PEG was precipitated in diethyl ether in an ice bath and was recovered by filtration. The precipitate was washed with diethyl ether and dried in vacuo. 100% acrylation, 1H NMR ($CDCl_3$): 3.6 ppm (394.38 H, PEG), 4.3 ppm (t 1.93 H, $-CH_2-C\underline{H}_2-O-CO-CH=CH_2$), 5.8 ppm (dd 1.00 H, $CH_2=C\underline{H}-COO-$), 6.1, 6.4 ppm (dd 2.08 H, $C\underline{H}_2=CH-COO-$) .

PEG-triacrylate

PEG-triacrylate (3500 Da) was prepared similarly from a three-armed PEG, the arms initiated from a central glycerol core, and was a gift of Neocrin Company (Irvine, CA, USA). MALDI-TOF mass spectrometry: 2900 Da. 100% acrylation, 1H NMR ($CDCl_3$): 3.6 ppm (83.97 H, PEG), 4.3 ppm (t 2.04 H, $-CH_2-C\underline{H}_2-O-CO-CH=CH_2$), 5.8 ppm (dd 1.04 H, $CH_2=C\underline{H}-COO-$), 6.1, 6.4 ppm (dd 1.95 H, $C\underline{H}_2=CH-COO-$).

PEG-dithiol

PEG-dithiol, mol wt. 3400 Da, was purchased from Shearwater Polymers (Huntsville, AL, USA) (supplier's analysis: 3387 Da, 97% substitution by 1H NMR, 100% substitution by UV, 3.8% disulfide by NMR and GPC).

PEG-divinylsulfone

PEG-divinylsulfone (PEG-diVS; PEG-bis-vinyl sulfone) (3400 Da) was purchased from Shearwater Polymers (Figure 2. 2a) or made from PEG-dithiol and divinyl sulfone (Figure 2. 2b). Divinyl sulfone (Fluka, 1.2 mL, 20 molar excess per thiol) was dissolved in triethanolamine buffer (25 mL, 50 mM triethanolamine, pH 8.0) and stirred at room temperature in a 100 mL round-bottom flask. Separately, PEG-dithiol (1.0 g) was dissolved in triethanolamine buffer (25 mL). As soon as the PEG-dithiol had dissolved, it was dripped into the stirring divinyl sulfone using a dropping funnel. The reaction was run for 1-2 hours at room temperature. The aqueous reaction solution was then twice extracted with diethyl ether (150 mL) at room temperature. The aqueous phase containing the PEG was freeze dried; the ether phase containing excess divinyl sulfone was neutralized with mercaptoethanol. The freeze dried product was dissolved in dichloromethane and filtered through paper. It was then precipitated by dripping into stirred diethyl ether (chilled in an ice bath) and the precipitate removed by filtration through paper. The white filter cake was washed with ether, collected, and dried in vacuo. The precipitation from dichloromethane was repeated once. The product white powder was then dissolved in water, sterile filtered (0.22 μm) and freeze-dried. 0.81 g, 97% vinylation, 1H NMR (CDCl3): 3.6 ppm (156.46 H, PEG), 4.0 pm (t 1.59 H, $-CH_2-SO_2-$), 6.2, 6.5 ppm (m 1.904 H, $C\underline{H}_2=CH-SO_2-$), 6.7 ppm (m 1.063 H, $CH_2=C\underline{H}-CO_2-$).

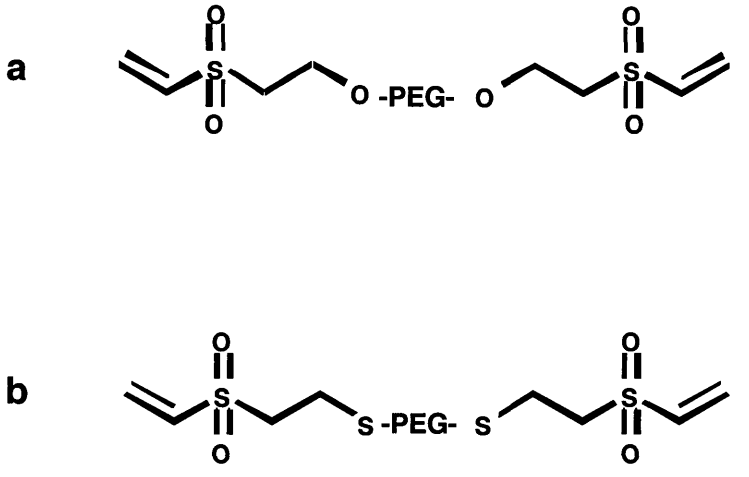

Figure 2. 2 Structures of PEG-divinylsulfones

a PEG-divinylsulfone from Shearwater Polymers
b PEG-divinylsulfone as synthesized according to methods herein.

Peptides

GCYKNRCYKNRCG-NH_2 and GCYKNRCYKNRCYKNRCG-NH_2 were synthesized using standard Fmoc solid phase peptide synthesis techniques according to Chapter 3.

Model kinetics

To determine the kinetics of reaction between the thiol and the acrylate in aqueous solution, PEG-monoacrylate was reacted with various amino acids. PEG-monoacrylate was reacted with the thiol of N-acetyl-L-cysteine (Calbiochem-Novabiochem, Laüfelfingen, Switzerland), the ε-amine of α-N-acetyl-L-lysine (Calbiochem-Novabiochem), and the α-

amine of glycyl-glycine (Fluka) in HEPES buffered saline (HEPES BS: 50 mM N-[2-Hydroxyethyl]piperazine-N'-[4-butanesulfonic acid] (13.02 g/L)), pH 7.4 at 37°C. The rate of consumption of thiols was measured using Ellman's reagent (5,5'-dithio-bis-(2-nitrobenzoic acid), Sigma, St. Louis, MO, USA). 40 mg of Ellman's reagent was dissolved in 10 mL of 0.1 M phosphate buffer, pH 8.0. 100 µl of Ellman's reagent in solution was added to 3 ml of 0.1 M phosphate buffer, pH 8.0 containing 0.05 – 0.15 µmol thiol-containing compound. The thiol concentration was measured spectro-photometrically using an extinction coefficient of 14,150 at 412 nm.[22] The rate of amine consumption was followed with fluorescamine (Sigma).[23] Fluorescamine was dissolved in acetone at a concentration of 1.5 mg/mL and was used within 4 hr. To 890 µL of 200 mM sodium tetraborate buffer, pH 9.0, 10 µL of sample and 100 µL of fluorescamine solution were added (such that the final amine concentration was 0 - 67 nmol/ml) and mixed promptly. Fluorescence was measured (λ_{ex} = 405 nm, λ_{em} = 475 nm) using a Waters 2690 pump and autosampler, a Waters 474 fluorescence detector, and Waters Millennium32 software package (Waters, Milford, MA, USA).

Rheometry

The gelation behavior of the material precursors was studied using small-strain oscillitory shear experiments with a Bohlin CVO 120 High Resolution rheometer and a plate-plate geometry in a humid atmosphere. Amplitude sweeps of the precursors in solution were first performed in order to determine the frequency and strain parameters where one could operate within the linear viscoelastic regime of the precursors. Precursor solutions (60 µL total) were applied to the bottom plate. The upper plate (2 cm diameter) was then immediately lowered to a measuring gap of 0.1 mm. After a short pre-shear period, the

dynamic oscillating measurement was started using an autostress mode. The evolution of the storage and loss moduli (G′ and G″, respectively) and phase angle (δ) at a constant frequency of 2.5 Hz were recorded. Also, a delay time was recorded by starting a timer at the time of precursor mixing and stopping the timer when the first data point was recorded by the rheometer; this delay time was used to correct the gel point recorded by the instrument. All measurements were made at 25 °C unless otherwise indicated.

For rheometric experiments with acrylate-containing precursors, 54 μL HEPES BS were added to 2.4 mg PEG-triacrylate. This solution was then added to 3.6 mg PEG-dithiol and promptly mixed immediately prior to loading the sample onto the rheometer and initiating the data recording. For vinylsulfone-containing precursors, 54 μL of HEPES BS were added to 6 mg PEG-diVS, and this solution was promptly mixed with 1.8 mg tricysteine peptide and loaded onto the rheometer. PEG concentration was held constant between the two systems.

Cytocompatibility

Toxicity of PEG-diVS was tested as a function of PEG-diVS concentration and exposure time. Human foreskin fibroblasts (Clonetics, Walkersville, MD, USA) were seeded at 3,000 cells/well in flat-bottomed 96 well plate wells. They were cultured for 2 d. 132 mg PEG-diVS was dissolved to make 1 mL in serum-containing medium and filter sterilized (100%). Serial dilutions were made to obtain 10%, 1%, 0.1%, and 0.01% solutions. Cell culture medium was changed completely to apply the PEG-diVS solutions (100 μL/well). Cells were exposed to the 100% concentration for 0, 10, and 30 min and to all concentrations for 1 hr, then washed three times with fresh serum-containing medium and cultured until the first wells approached confluence. Cell morphologies were observed and then the cells

passaged to 48 well plate wells. Cells were cultured until just below confluence and then passaged to 24 well plate wells. Just before cells approached confluence, they were trypsinized and the cell number in each well counted using a hemacytometer. Each condition was performed in quadruplicate.

Statistics

Values are given as means ± average deviations. P values were calculated using a two-tailed, non-paired Student t test.

RESULTS

Qualitative kinetics using PEG-monoacrylate

The rate of reaction of PEG-monoacrylate with a thiol-containing compound was much more rapid than the rates of reaction of PEG-monoacrylate with amine-containing compounds chosen to model thiol and amine reactivity in proteins. At pH 7.4 and 37°C, selected to model physiological conditions for *in situ* material formation, the pseudo first order half-life of the addition of the thiol of N-acetyl-L-cysteine to the acrylate of PEG-monoacrylate was approximately 7 min at a concentration of PEG-monoacrylate of 0.1 g/mL (a concentration relevant for gel formation) and an initial ratio of 10 acrylates to 1 thiol. By comparison, the half-life of the second-order reaction between thiols to form disulfide bonds was 11 hr at the same concentration of N-acetyl-L-cysteine (0.33 mg/mL). The pseudo first-order half-lives of the addition of the α-amine of glycyl-glycine and the ε-amine of α-N-acetyl-L-lysine were 5 hr and 21 hr respectively with an initial concentration of PEG-

monoacrylate of 0.1 g/mL and an initial ratio of 10 acrylates to 1 amine. The reaction between PEG-acrylates and the protein albumin was also shown to be slow.[24]

Gel formation kinetics and gel mechanical properties

Hydrogels were formed according the schemes shown in Figure 2. 3.

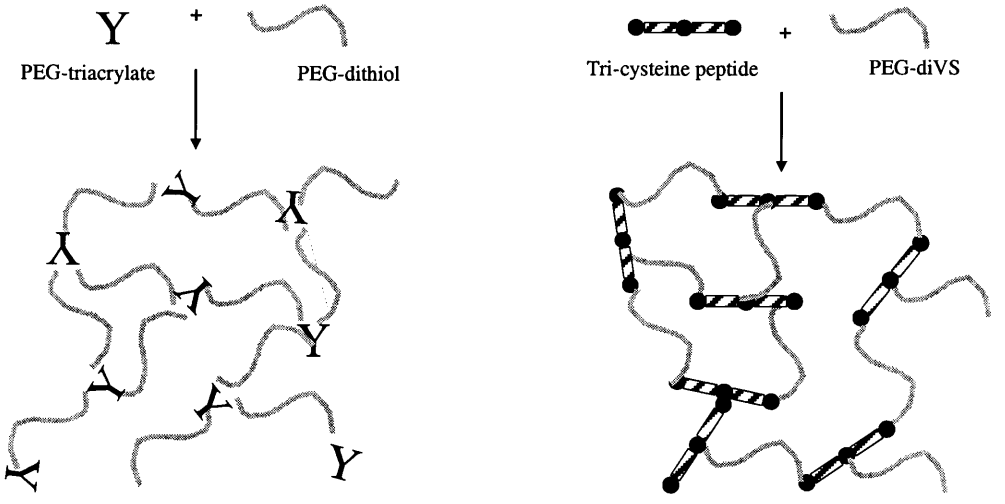

Figure 2. 3 Cartoon of hydrogel network formation

Small strain oscillatory shear experiments were performed to probe the viscoelastic behavior of gel precursor solutions as they transformed as a function of time into viscoelastic solids. Dynamic strain and induced stress were measured and instantaneous values recorded. Ratios of the instantaneous values were used to determine the complex shear modulus, G*, according to Hooke's law. G* is the sum of an elastic component called the storage modulus, G′, and a viscous component called the loss modulus, G″: $G^* = G' + iG''$. The two components of G* are related through the phase difference between the stress and strain, the phase angle, δ: $G' = G^* \cos(\delta)$ and $G'' = G^* \sin(\delta)$. The gel point was defined as G′ = G″ ($\tan(\delta) = 1$ or $\delta = 45°$).[25]

Figure 2. 4 shows a sample evolution of the storage modulus, loss modulus, and phase angle for gelation of PEG-triacrylate and PEG-dithiol at a total PEG concentration of 10% at pH 7.4 and 25°C. The gel point occurred in approximately 15 minutes but the mechanical properties continued to evolve for approximately 40 min leading to an ultimate G′ on the order of 10 kPa. The slow approach of the mechanical properties to their final state is due to hindered mobility of unreacted chains after the gel point.

For all gels formed by PEG-multiacrylates and PEG-thiol (varying precursor concentration and PEG-acrylate functionality) or by PEG-diVS and a tri-cysteine peptide, the storage moduli averaged 10,800 ± 2700 Pa upon completion of the cross-linking reaction and prior to swelling. This corresponded to a Young's modulus (E) of approximately 30 kPa ($E = 2(1+\mu)G'$, where μ = Poisson's ratio assumed to be 0.5 for cross-linked gels. Values of the storage modulus were typically 10-100 fold higher than those of the loss modulus. Final phase angles were approximately 5-10° (0° representing a pure solid and a completely in-phase response of stress and strain).

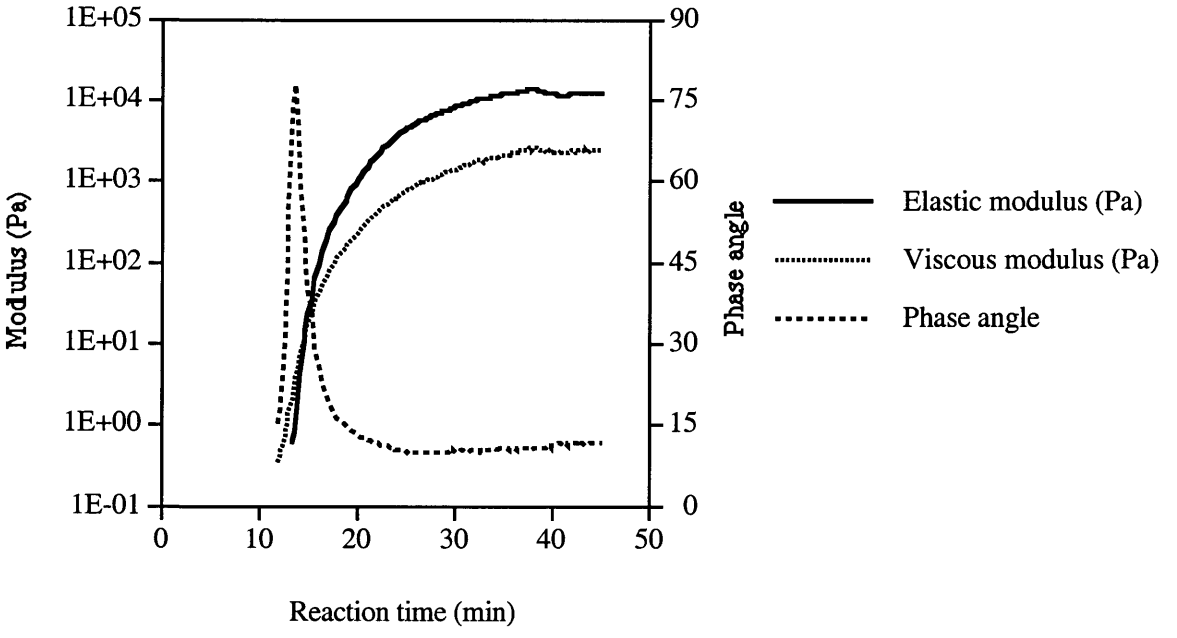

Figure 2. 4 Characteristic rheometric observation of gel formation
10% PEG-triacrylate and PEG-dithiol, pH 7.4, 25°C.

The gel point was observed to be a function of pH over a pH range of interest for forming materials in contact with cells and tissues, pH 7.0-8.2. For the combination of PEG-triacrylate and PEG-dithiol, the gel point at pH 7.0 was 22 ± 1 min and decreased linearly with increasing pH to 6 ± 1 min at pH 8.2. Using the combination of PEG-diVS and a peptide containing three reduced cysteines, the gel point was determined to be less than that for the acrylate system, 5 ± 1 min at pH 7.0 and 2 min at pH 8.2. Using the combination of PEG-diVS and a peptide containing four reduced cysteines, gelation was too rapid at 25 °C and all pH (7.0-8.2) to evaluate gelation behavior rheometrically. PEG concentrations were kept constant for the two systems; however, due to the molecular weight differences of the

cross-linking species, this lead to 50% higher concentration of reactive termini in the vinylsulfone system, the most likely reason for the faster gelation.

The gel point was also significantly affected by temperature. For the combination of PEG-triacrylate and PEG-dithiol at pH 7.4, the gel point occurred at 16 ± 1 min at 25 °C and at 10 ± 2 min at 37 °C.

Cytocompatibility

Fibroblasts exposed for 10 min to PEG-diVS at 132 mg/mL (the concentration used to make gels) did not show any decrease in proliferative capacity nor change in phenotype following passaging. However, cells exposed for 30 min showed a 39% decrease in proliferative capacity ($p < 0.05$) (Figure 2. 5). When exposed for 1 hr to different concentrations of PEG-diVS, only those cells exposed to the maximal concentration (132 mg/mL) of PEG-diVS showed a significant change (72% decrease) in proliferative capacity. All other concentrations (0.01-10% or 13.2 μg/mL – 13.2 mg/mL) were not statistically different from control cells which were not exposed to PEG-diVS (Figure 2. 6).

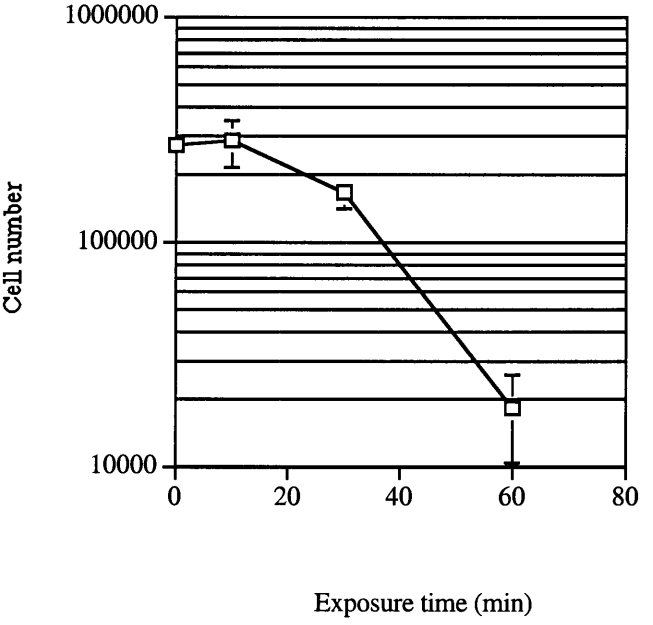

Figure 2. 5 Effect of exposure time to PEG-diVS on fibroblasts

Cell number following exposure to PEG-diVS (132 mg/ml, the starting concentration for gel formation) and two cell passages.

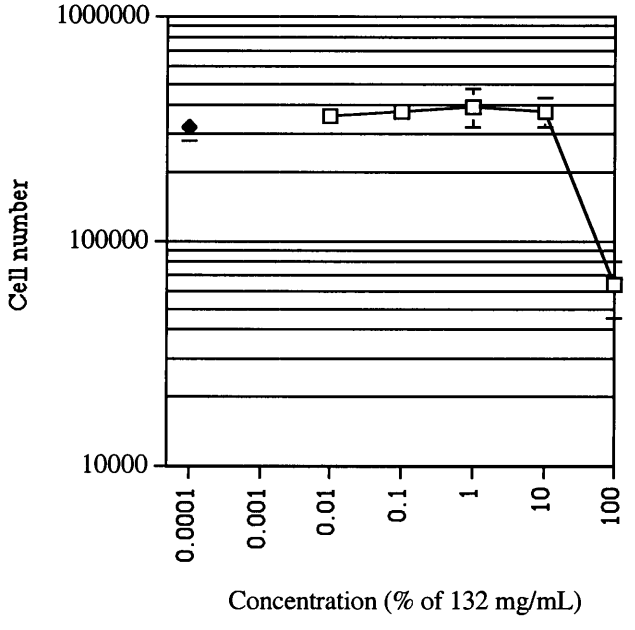

Figure 2. 6 Concentration dependence of PEG-diVS cytotoxicity

Following exposure for 1 hr to dilutions of the concentration of PEG-diVS used to form materials and two cell passages. The filled diamond represents controls with no PEG-diVS.

DISCUSSION

Natural extracellular matrices are hydrogels assembled and stabilized by a complex set of physicochemical processes and covalent cross-links. They provide mechanical support for cells and tissues and adhesive substrates for cells, they are enzymatically degradable, and they sequester growth factors. In an attempt to mimic some characteristics of natural extracellular matrices using synthetic starting materials, covalently cross-linked hydrogels were formed by conjugate addition reactions involving activated PEGs or activated PEGs and peptides containing reduced thiols. The gels were formed from aqueous precursor solutions, which is desirable for forming materials in contact with proteins and cells. The gels formed in seconds to minutes, which are surgically relevant time scales. Furthermore, the gels were formed at pH values, specifically pH 7.0-8.2, of physiological relevance for *in situ* formation of biomaterials. Below pH 8, the reactions are selective enough to have low rates of side reaction with extracellular molecules, predominantly proteins. These low rates of side reaction with proteins were demonstrated with bovine serum albumin.[24]

Activated PEGs containing vinylsulfones were selected not only for their thiol selectivity but also for the hydrolytic stability of the resulting covalent bond formed by their reaction with thiols.[12] Hydrolytic stability was desirable for the work presented in the following chapters, specifically in order to test the hypothesis that synthetic materials can be designed to be responsive to cellular activity such as cell migration.

The PEG-divinylsulfone used was shown not to be cytotoxic at concentrations and time scales used to form materials in contact with cells and tissues. However, cytotoxicity of PEG-divinylsulfone at longer exposure times should not be ignored. Although no

divinylsulfone could be observed in proton NMR spectra, the toxicity may be due to trace quantities of divinylsulfone used to prepare the PEG-divinylsulfone. Alternatively, the toxicity may be due to the ability of PEG to interact with cell membranes, albeit usually at higher concentrations,[26, 27] or due to endocytosis.[21] Closer evaluation of precise mechanisms for the cytotoxicity of PEG-divinylsulfone is recommended.

As an alternative to using vinylsulfone, one can use other Michael-type acceptors with potentially fewer cytotoxic effects and perhaps even higher thiol-selectivity. Toward this end, functional groups such as acrylates,[24] acrylamides,[28] and quinones[29] are being studied.

In this dissertation, the mechanical properties of hydrogel materials were held as constant as possible in order to study the effects of variables and design parameters, e.g., cell adhesivity and enzymatic degradation, other than mechanical properties. However, with the same conjugate addition cross-linking approach but with different precursors, molar ratios of reactants, and solvents, one can obtain a wide spectrum of materials and mechanical properties. For example, with lower molecular weight and more hydrophobic precursors, such as PEG570-diacrylate and pentaerythritol tetrakis(3-mercaptopropionate) in N-methyl-2-pyrrolidinone, one can obtain materials that withstand compression forces of 12 MPa and are of interest for load-bearing sites of injury, such as the vertebral disc.[30]

REFERENCES

[1] Hubbell JA. In situ material transformations in tissue engineering. *MRS Bull.* **21**, 33-35 (1996).

[2] Slepian MJ. Polymeric endoluminal paving. *Cardiol. Clin.* **12**, 715-737 (1994).

[3] Chen GH & Hoffman AS. Graft-copolymers that exhibit temperature-induced phase-transitions over a wide range of pH. *Nature* **373**, 49-52 (1995).

[4] Yoshida R, et al. Comb-type grafted hydrogels with rapid de-swelling response to temperature changes. *Nature* **374**, 240-242 (1995).

[5] Jeong B, Bae YH, Lee DS & Kim SW. Biodegradable block copolymers as injectable drug-delivery systems. *Nature* **388**, 860-862 (1997).

[6] Petka WA, Harden JL, McGrath KP, Wirtz D & Tirrell DA. Reversible hydrogels from self-assembling artificial proteins. *Science* **281**, 389-392 (1998).

[7] Galaev IY & Mattiasson B. 'Smart' polymers and what they could do in biotechnology and medicine. *TIBTECH* **17**, 335-340 (1999).

[8] Moghaddam MJ & Matsuda T. Molecular design of three-dimensional artificial extracellular matrix: photosensitive polymers containing cell adhesive peptide. *J. Polym. Sci. A.* **31**, 1589-1597 (1993).

[9] Pathak CP, Sawhney AS & Hubbell JA. Rapid photopolymerization of immunoprotective gels in contact with cells and tissues. *JACS* **114**, 8311-8312 (1992).

[10] Sperinde JJ & Griffith LG. Synthesis and characterization of enzymatically-cross-linked poly(ethylene glycol) hydrogels. *Macromolecules* **30**, 5255-5264 (1997).

[11] Zhao X & Harris JM. Novel degradable poly(ethylene glycol) hydrogels for controlled release of protein. *J. Pharm. Sci.* **87**, 1450-1458 (1998).

[12] Morpurgo M, Veronese FM, Kachensky D & Harris JM. Preparation and characterization of poly(ethylene glycol) vinyl sulfone. *Bioconjug. Chem.* **7**, 363-368 (1996).

[13] Jain RA. The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. *Biomaterials* **21**, 2475-2490 (2000).

[14] Sung YK & Kim SW. Advances in biodegradable polymers for drug delivery systems. *Korea Polymer Journal* **8**, 199-208 (2000).

[15] West JL. In situ formation of polymer matrices for localized drug delivery. *Therapeutic Protein and Peptide Formulation and Delivery*. Shahrokh Z (ed.) (American Chemical Society, Washington, DC, 1997).

[16] Hubbell JA. Synthetic biodegradable polymers for tissue engineering and drug delivery. *Curr. Opin. Solid. State & Mat. Sci.* **3**, 246-251 (1998).

[17] Brinkley M. A brief survey of methods for preparing protein conjugates with dyes, haptens, and cross-linking reagents. *Bioconj. Chem.* **3**, 2-13 (1992).

[18] Stryer L. *Biochemistry*. (W.H. Freeman and Company, New York, 1995).

[19] Dearfield KL, Harrington-Brock K, Doerr CL, Rabinowitz JR & Moore MM. Genotoxicity in mouse lymphoma cells of chemicals capable of Michael addition. *Mutagenesis* **6**, 519-525 (1991).

[20] Abuchowski A, McCoy JR, Palczuk NC, van Es T, & Davis FF. Effect of covalent attachment of poly(ethylene glycol) on immunogenicity and circulating life of bovine liver catalase. *J. Biol. Chem.* **252**, 3582-3586 (1977).

[21] Harris JM. Introduction to Biotechnical and Biomedical Applications of Poly(Ethylene Glycol). *Poly(Ethylene Glycol) Chemistry Biotechnical and Biomedical Applications* Harris JM (ed.) (Plenum Press, New York, 1992).

[22] Riddles PW, Blakeley RL & Zerner B. Reassessment of Ellman Reagent. *Methods in Enzymology* **91**, 49-60 (1983).

[23] Udenfriend S, et al. Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science* **178**, 871-872 (1972).

[24] Elbert DE, Pratt AB, Luetolf MP, Halstenberg S & Hubbell JA. Protein delivery from materials formed by self-selective conjugate addition reactions. *J. Control. Release* (in review).

[25] Ross-Murphy SB. Rheological characterization of gels. *J. Texture Studies* **26**, 391-400 (1995).

[26] Lentz BR. Polymer-induced membrane fusion: potential mechanism and relation to cell-fusion events. *Chemistry & Physics of Lipids* **73**, 91-106 (1994).

[27] Lore AB et al. Rapid induction of functional and morphological continuity between severed ends of mammalian or earthworm myelinated axons. *J. Neurosci.* **19**, 2442-2453.

[28] R. Schoenmakers, D. Elbert, P. van de Wetering & J. Hubbell, unpublished results.

[29] N. Tirelli et al., unpublished results.

[30] B. Vernon, N. Tirelli, D. Haldimann & J. Hubbell, unpublished results.

CHAPTER 3

PLASMIN-SUBSTRATE KINETICS AND PLASMIN-DEGRADABLE HYDROGELS

A novel peptidyl plasmin substrate was designed to be highly water-soluble and to contain reactive side chains (cysteine thiols) which would participate in conjugate addition reactions. Plasmin-substrate kinetics were measured for the peptide as were the kinetics when poly(ethylene glycol) (PEG) was tethered to a residue near each end of the peptide by addition reaction with its cysteine side chains. Physiological turnover numbers were observed in both cases. This was particularly important in the case of the PEG-tethered peptide which was used, albeit a soluble substrate, to mimic the peptide and enzyme environment in hydrogels containing the peptide sequence. A multimer of the peptidyl plasmin substrate was synthesized to contain three cysteine residues and two plasmin degradable sequences, one between each pair of cysteines. The multimeric peptide was cross-linked into hydrogels using activated, thiol-specific PEGs. The gels were degraded by plasmin in 4-5 hr but were not observed to be degraded by collagenase. Control peptides and gels made from them were not observed to be degraded by plasmin.

INTRODUCTION

To date, most synthetic biomaterials degrade on their own chemical time scales. However, for materials to be healing-responsive, they must instead react to cells and the biochemical mechanisms used by cells to migrate within and remodel matrices. These mechanisms are enzymatic and mediated primarily by two classes of proteolytic enzymes, serine proteases and matrix metalloproteinases (MMPs). Plasmin is the predominant matrix-degrading enzyme of the serine protease family and mediates the removal of the provisional fibrin matrix formed at sites of tissue injury. It is used ubiquitously by different cell types, including fibroblasts, macrophages, endothelial cells, and neurons. Hence plasmin was the enzyme of interest targeted in the design of the synthetic biomaterials described herein.

Design criteria

Design of a plasmin substrate cross-linker was subject to a number of constraints. The substrate should contain Michael acceptors or Michael donors in order to create material cross-links by conjugate addition reactions (Chapter 2). The cross-linker should be degradable by plasmin and have a way to be modified in order to make nondegradable control cross-linkers without significant change in cross-linking behavior, mechanical properties, etc. The demand for plasmin-degradability implied that the substrate should be a peptide or an ester of lysine or arginine. Furthermore, the peptide should contain at least one lysine for plasmin(ogen) to bind as required for plasmin to cleave its substrate.[1] The cross-linker should be soluble in relatively high concentrations (> 30 mg/mL) in order to form the material, and the ideal solvent would be an aqueous biological buffer solution.

Plasmin specificity

Plasmin is a proteinase (endopeptidase) which means that it cleaves peptide bonds within peptidyl substrates and not at the carboxy- or amino-termini of peptides and proteins. It cleaves only peptide bonds carboxy-terminal to basic residues, Lys- and Arg-. In this respect plasmin is similar to the digestive enzyme trypsin although plasmin has a higher specificity and lower activity.[2] Plasmin does not hydrolyze all Lys- and Arg- bonds but cleaves approximately 25% of them in its natural substrates – fibrinogen, fibrin, fibronectin, laminin – and has higher specificity for Lys-. Dissolution of fibrin does not require cleavage of Arg-. Plasmin's substrate specificity depends on the amino acid residues surrounding the Lys- or Arg- bond (Table 3. 1).[3] The rate of plasminolysis is significantly affected by P4, P2, P1, P1', and P2' amino acid residues;[4] in contrast, plasmin is relatively insensitive to the residue in the P3 substrate position.[5] Sequences of two or more adjacent basic residues are relatively resistant to plasmin but are susceptible to degradation by other serine proteases such as trypsin.[2]

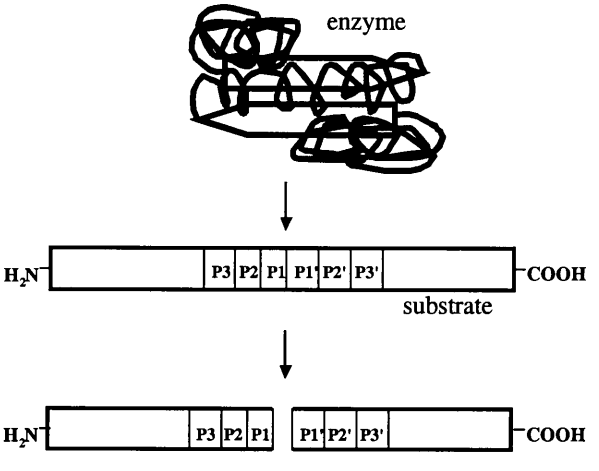

Figure 3. 1 P nomenclature for peptidyl enzyme substrates

Table 3. 1 Plasmin-substrate specificity

P2	P1	P1'	P2'	Effect
Tyr, Phe, Trp				enhance hydrolysis
Lys, Arg, His				slow hydrolysis
	Lys, Arg			required for hydrolysis
		Ser, Thr		enhance hydrolysis
		Pro		prevents hydrolysis
			Gly, Ala, Val	enhance hydrolysis
			Ser, Thr, Glu, Asp	slow hydrolysis

Plasmin-substrate kinetics have been reported for a number of synthetic peptidyl substrates.[6, 7, 8, 9, 10] However, values are reported only for variations in the sequence of amino acid residues amino-terminal to the Lys- or Arg- cleavage site. This focus is at least in part due to the p-nitroanilide (pNA) assay commonly used as well as more recent use of support-bound combinatorial library techniques.[5] Synthetic plasmin substrates based on the early plasmin-substrate kinetics work are commercially available for spectroscopic determinations of plasmin activity (Table 3. 2).

Table 3. 2 Some commercially available plasmin substrates

P3	P2	P1	P1'	Product	Source
Tosyl-Gly	Pro	Lys	pNA	Chromozym-PL	Roche
*D*Val	Leu	Lys	pNA	S-2251	Chromogenix
*D*Val	Phe	Lys	pNA	S-2390	Chromogenix
Pyro-Glu	Phe	Lys	pNA	S-2403	Chromogenix
*D*Ala	CHT	Lys	pNA	Pefachrome PL	Pentapharm

Substrate design

Because the well-studied plasmin substrates, such as the commercially available ones, contain relatively hydrophobic residues and no residues carboxy-terminal to the lysine, native plasmin substrates in fibrin(ogen) (Table 3. 3) were evaluated as potential sources of a plasmin substrate for the material cross-linker. Plasmin has been reported to cleave

fibrin(ogen) at as few as 4-9[11] sites and as many as 31[3] and 75[2] sites although the number required for clot dissolution is approximately 8-9.[12]

Table 3. 3 Plasmin cleavage sites in fibrin(ogen)*

P3	P2	P1	P1'	P2'	P3'	Chain P1	Reference
Leu	Ile	Lys	Met	Lys	Pro	α 206	12, 13
Asn	Phe	Lys	Ser	Gln	Leu	α 219	13
Glu	Trp	Lys	Ala	Leu	Thr	α 230	13
Thr	Gln	Lys	Lys	Val	Glu	β 53	13
Leu	Ile	Lys	Ala	Ile	Gln	γ 62	14, 15
Arg	Gln	Lys	Gln	Val	Lys	β 130	12
Ser	Arg	Lys	Met	Leu	Glu	γ 88	12
Ser	Tyr	Lys	Met	Ala	Asp	α 583	16
Gln	Val	Lys	Asp	Asn	Glu	β 133	15
Thr	Leu	Lys	Ser	Arg	Lys	γ 85	12, 14
Tyr	Gln	Lys	Asn	Asn	Lys	α 78	14
Gln	Met	Arg	Met	Glu	Leu	α 239	13
Gly	Tyr	Arg	Ala	Arg	Pro	β 42	12, 14
Asn	Asn	Arg	Asp	Asn	Thr	α 104	12, 15
Tyr	Asn	Arg	Val	Ser	Glu	α 110	12
Gly	Phe	Arg	His	Arg	His	α 491	16
Gly	Pro	Arg	Val	Val	Glu	α 19	14

*Lys- and Arg- cleavage sites are listed separately. Within each group, the cleavage sites are listed approximately in the order in which they are cleaved upon exposure of fibrin(ogen) to plasmin.

Lys was favored over Arg in the P1 position since Lys is kinetically favored by plasmin and is required for plasmin(ogen) binding. Lys also confers substrate specificity for plasmin compared to other serine proteases such as thrombin (Arg-Gly) and tPA and uPA (Arg-Val).

Consideration had to be taken regarding a method for incorporating Michael donors or acceptors. Since the substrate should be a peptide, cysteine residues are synthetically the most convenient source of thiols as Michael-donors. Thiols are preferable over other nucleophiles in peptides, such as Lys, for reasons discussed in Chapter 2 and because Lys is required for plasmin activity and degradability of the substrate. Therefore, Cys residues

were added to the substrate design, one at each end of the cleavage site to serve as future points of cross-linking. Predictions of relative peptide solubility were then made using the grand average of hydropathicity index (GRAVY)[17, 18] (Table 3. 4).

Table 3. 4 Hydropathic indices for Lys- cleavage sites in fibrin(ogen)

P3	P2	P1	P1'	P2'	P3'	P4'	GRAVY
Cys	Ile	Lys	Met	Lys	Pro	Cys	0.286
Cys	Phe	Lys	Ser	Gln	Leu	Cys	0.486
Cys	Trp	Lys	Ala	Leu	Thr	Cys	0.729
Cys	Gln	Lys	Lys	Val	Glu	Cys	-0.800
Cys	Ile	Lys	Ala	Ile	Gln	Cys	1.200
Cys	Gln	Lys	Gln	Val	Lys	Cys	-0.800
Cys	Arg	Lys	Met	Leu	Glu	Cys	-0.171
Cys	Tyr	Lys	Met	Ala	Asp	Cys	0.000
Cys	Val	Lys	Asp	Asn	Glu	Cys	-0.743
Cys	Leu	Lys	Ser	Arg	Lys	Cys	-0.614
Cys	Gln	Lys	Asn	Asn	Lys	Cys	-1.900

Relatively hydrophobic sequences (GRAVY > 0) were eliminated, and some amino acids were disfavored (Table 3. 5, italics). Met was considered undesirable for synthetic reasons, Gln was disfavored for solubility reasons,[19] and Lys outside of P1 was avoided in order to favor only one plasmin(ogen) binding and cleavage site per peptide.

Table 3. 5 Relatively hydrophilic cleavages sites in fibrin(ogen)

P3	P2	P1	P1'	P2'	P3'	P4'	GRAVY
Cys	*Gln*	Lys	*Lys*	*Val*	Glu	Cys	-0.800
Cys	*Gln*	Lys	*Gln*	*Val*	*Lys*	Cys	-0.800
Cys	Arg	Lys	*Met*	*Leu*	Glu	Cys	-0.171
Cys	Tyr	Lys	*Met*	*Ala*	Asp	Cys	0.000
Cys	*Val*	Lys	Asp	Asn	Glu	Cys	-0.743
Cys	*Leu*	Lys	Ser	Arg	*Lys*	Cys	-0.614
Cys	*Gln*	Lys	Asn	Asn	*Lys*	Cys	-1.900

Tyr was chosen for the P2 site since it is known to have a favorable effect on plasmin-substrate kinetics in that position (Table 3. 1). GRAVY values calculated for the remaining possible combinations of P1'-P3' amino acids indicated that Asn would be less favorable than Arg and that Ser would be less favorable for solubility than Asn and Asp; the positive effect of Ser in the P1' position on hydrolysis rates was not known at the time of substrate design. Among the remaining residues, no preference was given, and the substrate selected is given in Table 3. 6.

Table 3. 6 Cys-containing putative water-soluble plasmin substrate

P3	P2	P1	P1'	P2'	P3'	P4'	GRAVY
Cys	Tyr	Lys	Asn	Arg	Asp	Cys	-1.671

Cross-linker design

In order to form cross-linked networks, e.g., rather than linear block copolymers, the functionality of one monomer must be two or more, and the functionality of the other monomer must be three or more. Since PEGs are more readily available as bi-functional molecules, and peptides could be synthesized on demand, the peptide component of the hydrogels was selected as the monomer which would contain functionalities (thiols) of three or more.

Since the materials were designed to be degradable, it was also desirable to design them such that they would degrade into products (oligopeptide-PEGs) that could be excreted in the urine. Therefore the size of the degradation products should be smaller than renal threshold for PEG (< 20-50 kDa). Therefore, the materials were designed to be degradable between cross-links by placing a degradation site between each pair of cysteine residues, thus giving rise to a linear peptide repeat structure of $(\text{Cys-substrate})_n$. Thus, every increase in

functionality by 1 increased the peptide length by 6 residues (CYKNRD). It is known that microenvironment affects pK, and it was confirmed that the reactivity of the thiol was sensitive to its neighbors, such as Asp or Arg.[20] Therefore, upon multimerizing the plasmin substrate to form peptides of higher functionality, the aspartic acid (D) was eliminated in favor of shorter peptides and higher rates of addition and thus higher rates of gelation.

MATERIALS AND METHODS

Synthesis of peptides

Peptide sequences and their chemical modifications are given in Table 3. 7. Solid phase peptide synthesis was used to make the peptides. Di-cysteine peptides were synthesized on peptide acid resin (NovaSyn TGA or TGT, Novabiochem-Calbiochem AG, Laüfelfingen, Switzerland) and tri- and tetra-cysteine peptides were synthesized on peptide amide resin (NovaSyn TGR) using an automated peptide synthesizer (Pioneer, PerSeptive Biosystems, Framingham, MA, USA) and standard 9-fluorenyl-methyloxycarbonyl (Fmoc) chemistry.[21] Solvents were obtained from Applied Biosystems (Warrington, England). All other reagents were received from Novabiochem-Calbiochem unless otherwise noted. The amino acid derivatives used are listed in Table 3. 8. Following synthesis, peptide resin was washed with dichloromethane and dried *in vacuo* for 4 hr or more. Peptides from 1 g resin were cleaved and deprotected for 2.5 hr using 15 mL modified Reagent B: 88 parts trifluoracetic acid (TFA, Fluka, Buchs, Switzerland), five parts water, five parts phenol (Aldrich, Steinheim, Germany), and four parts triisopropylsilane (Aldrich). Resin was removed by filtration through fritted glass and washed with 2 resin volumes of TFA. Diethyl ether (300-500 mL, cooled in an ice bath) was added to the filtrate, also cooled in an ice bath.

The white precipitate was collected by filtration and washed with diethyl ether, all under a blanket of argon. The precipitate was dried *in vacuo*.

Peptides were purified using preparative C_{18} chromatography (Prep Nova-Pak 19 x 300 mm column, Waters, Milford, MA, USA; BioCAD 700E, PerSeptive Biosystems) using a gradient of acetonitrile against 0.1% TFA. Addition of reducing agents, dithiothreitol or tris[2-carboxyethyl]phosphine hydrochloride, did not significantly change the elution profiles and was not included in most cases. Eluted fractions were freeze-dried, and molecular masses confirmed with MALDI-TOF (Protein Service Laboratory, ETH Zürich; System 122, Perkin Elmer Biosystems, Foster City, CA, USA).

Table 3. 7 Peptide sequences and their chemical modifications

Peptide sequence	Design parameters	Abbreviation
GCYKNRDCG-COOH	di-cysteine plasmin-sensitive peptide	C2
GC(aa)YKNRDC(aa)G-COOH	di-cysteine plasmin-sensitive peptide, cysteines blocked	C2-aa
GC(PEG)YKNRDC(PEG)G-COOH	PEG-grafted di-cysteine plasmin-sensitive peptide	C2-PEG
GCY-*D*LysN-*D*ArgDCG-COOH	di-cysteine plasmin-insensitive peptide	*D*C2
GCYKNRCYKNRCG-NH2	tri-cysteine plasmin-sensitive peptide	C3
GCY-*D*LysN-*D*ArgCY-*D*LysN-*D*ArgCG-NH2	tri-cysteine plasmin-insensitive peptide	*D*C3

Table 3. 8 Amino acid derivatives used in peptide synthesis

Fmoc-*L*-amino acid(protecting group)-OH	Fmoc-*D*-amino acid(protecting group)-OH
Cys(Trt)	Lys(Boc)
Asp(otBu)	Arg(Pbf)
Gly	
Lys(Boc)	
Asn(Trt)	
Arg(Pbf)	
Tyr(tBu)	

Modification of peptides

Cysteine thiols in purified di-cysteine peptides were blocked. In one case, a small molecule, acetamide (aa), was incorporated using iodoacetamide (Fluka).[22, 23] In another case, PEG was incorporated using mono-methoxy-PEG 5000-2-(vinyl sulfonyl)ethyl ester (Fluka, PEG-monoVS). Iodoacetamide was dissolved (1.1 mg/mL) in HEPES buffered saline (HEPES BS: 50 mM N-[2-hydroxyethyl]piperazine-N'-[4-butanesulfonic acid] (13.02 g/L), 137 mM NaCl (8 g/L), pH 8.0). Peptide C2 was dissolved (1.0 mg/mL) in HEPES BS, and the two solutions were mixed in equal volumes with a 3:1 molar ratio of iodoacetamide to cysteine. The solution was stirred at room temperature. The extent of reaction was followed using analytical C_{18} chromatography (Nova-Pak, 3.9 x 150 mm column, Waters; Waters 2690 pump and autosampler; Waters 996 photodiode array detector; Waters Millennium32 software package) until completion (30-60 min). Preparative C_{18} chromatography was used to purify C2-aa and followed by MALDI-TOF to confirm the molecular mass. C2-PEG was prepared similarly. PEG-monoVS (30 mg) was dissolved in 1 mL HEPES BS. Peptide C2 (1 mg) was dissolved in 1 mL HEPES BS and added to the PEG-monoVS. The solution was stirred at room temperature. The extent of reaction was followed using analytical size exclusion chromatography (Shodex OHpak SB-803HQ, 8 x 300 mm column, 10 mM phosphate, 0.3 M NaCl, pH 7.4, 0.3 mL/min; as above, plus Waters 410 differential refractometer) until completion (1 hr).

Soluble plasmin-substrate kinetics

Initial substrate concentrations were based on estimated[7] or previously measured values of K_m such that substrate concentrations were $0.05*K_m$, $0.3*K_m$, $0.5*K_m$, and K_m, and initial substrate concentrations were 1000 molar excess over enzyme at estimated K_m values. Substrates were dissolved in 50 mM HEPES BS, pH 7.4, stirred, and heated in a 37 °C water bath. Plasmin (porcine, 1 U/mL, Sigma) or collagenase (bacterial, type 4, 1 U/mL, Worthington Biochemical Corporation, Lakewood, NJ, USA) dissolved in phosphate buffered saline (PBS: 2.7 mM KCl (0.2 g/L), 1.5 mM KH_2PO_4 (0.2 g/L), 137 mM NaCl (8.0 g/L), 8 mM $Na_2HPO_4 \cdot 7H_2O$ (2.16 g/L), pH 7.4) was added to the stirred substrates at 37° C. Divalent cations in the water used to prepare the buffers were sufficient in quantity for collagenase activity as determined by gelatin control solutions analyzed with polyacrylamide gel electrophoresis. Peptide samples (unmodified) were run in parallel without enzyme in order to determine rates of disulfide bond formation and identify side-products. Samples were withdrawn over time, and the enzymatic activity of the withdrawn samples was quenched by addition of acetic acid. Samples were stored at 4 °C until peak area analysis with analytical HPLC. C_{18} chromatography was used for C2, C2-aa, and *D*C2 samples; size exclusion chromatography was used for C2-PEG samples. Chromatographic methods were the same as those used to follow the extent of peptide modification during substrate preparation, above. Kinetic parameters, K_m and V_{max}, were determined using Lineweaver-Burk plots, and turnover number was calculated as V_{max}/initial enzyme concentration. Kinetic experiments were performed in duplicate, with the exception of C2-PEG due to insufficient quantity of plasmin from the same batch. Amino acid analysis was performed by Ragna Sack in the laboratory of Peter Hunziker, Biochemisches Institut, Universität Zürich.

Hydrogel formation

PEG-diVS (6 mg) was dissolved in 90 μL HEPES BS, pH 7.4, containing 10 mg/ml Blue dextran (Sigma, 2,000 kDa) and added to 1.8 mg tri-cysteine peptide. Two 50 μL aliquots of the resulting solution were applied to a glass slide which had previously been silylated (SigmaCote, Sigma, used according to manufacturer's instructions) to make it hydrophobic. A second silylated glass slide was placed on top of the first with two 700 μm-thick teflon spacers in between. The sandwich with the gel precursor in between was clamped together with ordinary office binder clips. The gel precursor spread to form a circle which had contact with the glass slides and with air only. The precursor was allowed to cure for 1 hour at 37 °C in a humid environment.

Gel disks were removed from their molds and placed in 5 mL PBS in 6-well plates. The gels were swollen at least 24 hr, which was longer than the approximately 4 hr needed to reach equilibrium. Some gels were stored at 37 °C in PBS containing 0.1% sodium azide to prevent growth of potential bacterial or fungal contamination during long term observation.

Hydrogel degradation

Images of swollen gels in PBS were taken (time zero). Then PBS was replaced by plasmin (0.1 U/mL x 5 mL), collagenase (10 U/mL x 5 mL), or PBS. Images were taken for 2 d. Enzyme solutions were not replenished. Images of gels were taken with a 4 mm x 4 mm grid in the background in order to better observe changes in gel size.[24]

Image processing

Digital images were acquired with a Stemi 2000-C microscope, KL1500 LCD light source, Konton Elektronik Progres 3008 scanning camera, and Adobe Photoshop 5.5 software (Zürich, Switzerland). Gaussian nine pixel blurring was applied in Photoshop to account for anisotropies in the gels caused by precipitates of Blue dextran. Quantitative grey scale analysis was then performed with a Leica QWin image analysis software package.

Statistics

Values are given as means ± average deviations. P values were calculated using a two-tailed, nonpaired Student t test.

RESULTS

Soluble plasmin-substrate kinetics

Peptide C2 was synthesized and purified. Its solubility in PBS was ≥ 45 mg/mL. Because the peptide and its putative plasmin-degradable substrate, YKNRD, are not found in any natural proteins described to date,[25] it was necessary to characterize the plasmin sensitivity of the peptide. This was done using homogeneous liquid phase enzyme-substrate kinetic studies. A K_m' was estimated to be 600 μM based on published K_m values for synthetic plasmin substrates.[7] Then the substrate at different concentrations, $0.05*K_m'$, $0.3*K_m'$, $0.5*K_m'$, $1.0*K_m'$, and $2.0*K_m'$, was exposed to plasmin at an enzyme concentration of $K_m'/1000$ so that the substrate was in a large excess. A pseudo-steady state hypothesis was assumed and Michaelis-Menten kinetic analysis were applied using a Lineweaver-Burk plot

to determine K_m and V_{max}. The assumed K_m' value was used for a first analysis in order to better determine K_m. Then the new calculated K_m was used for a follow-up experiment where the experimentally derived K_m was confirmed.

A linear Lineweaver-Burk plot was obtained for low substrate concentrations ($\leq K_m$), and deviation from linearity was observed > K_m. This nonlinearity at high substrate concentrations has previously been observed for plasmin[11] and other serine proteases.[26, 27] The effect is attributed to allosteric effects, binding of more than one substrate molecule to the enzyme, and "substrate activation" at high substrate concentrations. This allosteric effect makes determination of kinetic parameters more complex. In this work, K_m and V_{max} values were calculated only in the low concentration linear regime and are therefore valid only for concentrations below the measured K_m.

In order to determine the kinetic effect of modifying the peptidyl plasmin substrate, e.g., for incorporation into a material, and to minimize side reactions due to disulfide bond formation, the side chains of the cysteine residues of peptide C2 were covalently modified. In one case a small molecule, acetamide, was incorporated using iodoacetamide. In another case, PEG was incorporated using PEG-monoVS.

Table 3. 9 lists the K_m, V_{max}, and turnover numbers for the plasmin substrates studied. The values for C2 and C2-aa are typical of small plasmin substrates. The turnover number for C2-PEG was of the same order of magnitude (5 – 25/s) as plasmin for soluble fibrin.[28]

Table 3. 9 Plasmin-substrate kinetic parameters

substrate	K_m (μM)	V_{max} (μM/min)	Turnover (1/s)
C2	446 ± 5	11.3 ± 0.2	319 ± 7
C2-aa	188 ± 58	6.3 ± 0.6	178 ± 16
C2-PEG	640	1.3	5

Plasmin-degradability of hydrogels formed by conjugate addition

Blue dextran is a large molecular mass carbohydrate polymer with a covalently attached dye. Under the gel formation conditions used, Blue dextran did not participate in the Michael-type addition which cross-links the gel; because of its size, Blue dextran was trapped inside the network pores as the network formed. The Blue dextran did not diffuse out of gels unless the gels' degree of cross-linking was reduced, e.g., by proteolytic degradation. Gels formed with peptide C3 and exposed to plasmin (0.1 U/mL x 5 mL) were degraded in 4-5 hr (Figure 3.2; a: 0 hr; b: 1 hr; c: 2 hr; d: 3 hr; e: 4 hr; f: 5 hr; 4 mm x 4mm grid). Gels exposed to collagenase at a concentration 100-fold higher than the plasmin were not degraded during the duration of the experiment (2 days). Gels stored in PBS containing 0.1% sodium azide at 37 °C were stable and did not demonstrate any macroscopic change in mechanical properties for at least 4 months. Gels formed with peptide *D*C3 and exposed to plasmin (2 U/mL x 5 mL) were not degraded during the duration of the experiment (2 days).

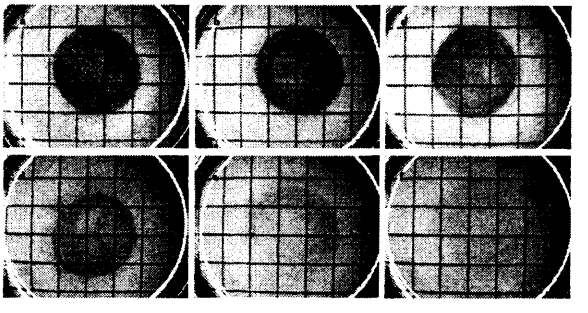

Figure 3. 2 Synthetic hydrogels degrade in the presence of plasmin

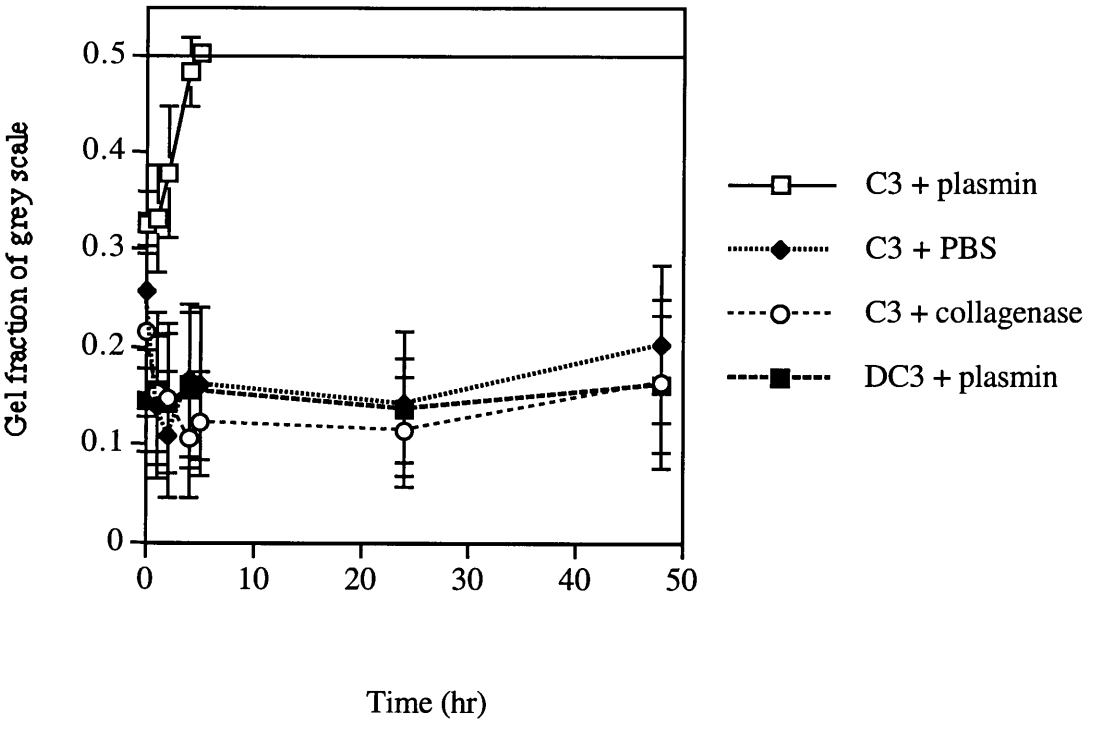

Figure 3. 3 Grey scale analysis of gel degradation

Grey scale analysis was used to quantify gel degradation. At 0.5, the grey scale of the gel was equal to that of the solution.

DISCUSSION

A novel di-cysteine peptide plasmin substrate was designed and synthesized. PEG was conjugated to it by conjugate addition. The resulting PEG-conjugated peptide was degradable by plasmin with a turnover number of 5/s. This rate is similar to that reported for fibrin-bound plasmin and its substrate, fibrin. In that case, a turnover number of 5-25/s has been reported.[28] The fact that the synthetic substrate has a turnover number of the same order of magnitude as plasmin for one of its natural substrates implies that it might be useful within a matrix for cellular ingrowth and wound healing.

The di-cysteine peptide and a tri-cysteine multimer of it are highly water soluble (each ≥ 45 mg/mL). Solubility of ≥ 28 mg/mL was required in order to form a hydrogel

containing 10% PEG (mass/volume) and equimolar thiols and PEG-conjugated Michael acceptors (vinylsulfones).

The di-cysteine peptides were synthesized as peptide acids, but the tri-cysteine (and tetra-cysteine peptides synthesized later, Chapter 4) were synthesized as peptide amides. This was done intentionally for two reasons. First, the peptide acids were strongly acidic in aqueous solution, and this acidity was not attributed to residual TFA from the cleavage, deprotection, and purification steps. Repeated freeze drying from large volumes of neutral solution could not reduce the acidity of the peptides. Solutions with high peptide concentration for material formation were extremely difficult to buffer. Buffers normally considered to be strong biological buffers – 100 mM PBS, 100 mM histidine buffer, and 100 mM HEPES BS at pH 8 – were not strong enough buffers. Triethanolamine solutions were used; however, the pH was very sensitive to relatively small changes in concentration of triethanolamine, and therefore reproducibility with respect to pH of material formation was a problem with peptide acids.

The second reason to use peptide amides instead of peptide acids was to favor the formation of the thiolate anion by control of the cysteine microenvironment and thus speed the rate of material formation relative to the peptide acid. For this same reason, and to keep the multimeric peptides as small as possible, the aspartic acid used in the di-cysteine peptides was eliminated in tri- and tetra-cysteine peptides.

The plasmin substrate used, YKNR, contained an arginine as well as a lysine that could potentially be cleaved by plasmin. In an attempt to determine the relative rates of cleavage of the two putative degradation sites, Lys- and Arg-, enzyme substrate kinetic studies were performed on peptide C2 with single *D*Lys or *D*Arg substitutions, as opposed to

the double substitution in peptide *D*C2. No plasmin cleavage of either peptide was observed in 27 hr indicating an unfavored structure in both cases as a result of the *D* stereoisomers. *D* stereoisomers of amino acid residues amino-terminal to P1 in synthetic substrates are usually not unfavorable for plasmin activity. In fact, they are often observed to be beneficial[7] and are used in the P3 positions of some commercially available chromogenic plasmin substrates (Table 3. 2).

Amino acid analysis of the plasmin degraded C2 peptide confirmed the presence of the GCYK fragment and cleavage of the Lys- bond. None of the putative degradation product NR could be observed, and the results were inconclusive regarding the presence of the putative DCG fragment due to the nature of the sequence. With C_{18} chromatographic analysis of the degradation products, one major product was observed. A minor product peak corresponded to that observed during disulfide bond formation of the peptide under the same conditions but in the absence of enzyme. As a combined result of the amino acid analysis and chromatographic observations, the Lys- bond was assumed to be the preferred plasmin cleavage site within the peptide GCYKNRDCG; whether the Arg- bond is cleaved upon long term exposure to plasmin can not be ruled out.

Tri-cysteine peptides were synthesized and used as cross-linkers to form viscoelastic solids by conjugate addition with activated PEG (PEG-divinylsulfone). The resulting hydrogels formed from a plasmin-degradable cross-linking peptide could be specifically degraded by the targeted enzyme, plasmin. Therefore, the materials can be degraded by one of the same mechanisms used by cells for migration and wound healing. By exchanging the plasmin substrate used here (YKNR) for another peptide sequence, one can form similar materials which are degradable by other enzymes used in wound healing, such as MMPs.[20]

One can also synthesize protease-insensitive materials by choosing proteolytically-stable amino acid sequences or using β- or *D*-amino acids (the latter described herein with *D*C2 and *D*C3). Such materials might be useful to encapsulate allogenic cells for transplantation or for use in extracorporeal devices such as a bioartificial liver.

Systematic study of the effect of enzyme-substrate kinetic parameters such as K_m on cell migration (Chapter 4) would be possible with materials described herein. For plasmin, only the amino-terminal side of the cleavage site has been quantitatively described. Therefore, one would have to synthesize a series of peptides including P' amino acids, perhaps according to the qualitative observations in Table 3. 1, characterize them kinetically, and then proceed to study the effects on cell migration and wound healing. With the MMPs, kinetic parameters for synthetic substrates of varying P4-P4' are well described,[29, 30] and one can directly start to evaluate the role of enzyme-substrate kinetic parameters on cell migration and wound healing.[20]

REFERENCES

[1] Bachmann F. The plasminogen-plasmin enzyme system. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice, Third Edition.* Colman RW, Hirsh J, Marder VJ & Salzman EW (eds). (J.B. Lippincott Company, Philadelphia, 1994).

[2] Wallén P & Iwanaga S. Differences between plasmin and tryptic digests of human S-sulfo-fibrinogen. *Biochim. Biophys. Acta* **154**, 414-417 (1968).

[3] Kiel B. *Specificity of proteolysis.* (Springer-Verlag, New York, 1992).

[4] Schechter I & Berger A. On the size of the active site in proteases. I. Papain. Biochem. Biophys. Res. Commun. 27, 157-162 (1967).

[5] Backes BJ, Harris JL, Leonetti F, Craik CS & Ellman JA. Synthesis of positional-scanning libraries of fluorogenic peptide substrates to define the extended substrate specificity of plasmin and thrombin. *Nature Biotechnology* **18**, 187-193 (2000).

[6] Claeson G, Aurell L, Karlsson G & Friberger P. Synthetic substrates and inhibitors for coagulation factors (ICTH). *Topics in hematology: proceedings of the 16th international congress of hematology, Kyoto.* 381-384 (1976).

[7] Cs.-Szabó G, Pozsgay M & Elödi P. Investigation of the substrate-binding site of human plasmin using tripeptidyl-p-nitroanilide substrates. *Thromb. Res.* **20**, 199-206 (1980).

[8] Okada Y et al. Synthesis of plasmin substrates and relationship between their structure and plasmin activity. *Int. J. Peptide Protein Res.* **27**, 79-85 (1986).

[9] Wijkmans JCHM, Nagel A & Bloemhoff W. A novel chromogenic plasmin substrate Isoval-Phe-Lys-pNA•HCl: a mild approach to the synthesis of *N*-α-peptidyl-L-lysine *p*-nitroanilides. *Lett. Pept. Sci.* **3**, 107-112 (1996).

[10] Butenas S, DiLorenzo ME & Mann KG. Ultrasensitive fluorogenic substrates for serine proteases. *Thromb. Haemost.* **78**, 1193-1201 (1997).

[11] Weinstein MJ & Doolittle RF. Differential specificities of thrombin, plasmin and trypsin with regard to synthetic and natural substrates and inhibitors. *Biochim. Biophys. Acta* **258**, 577-590 (1972).

[12] Hantgan RR, Francis CW & Marder VJ. Fibrinogen structure and physiology. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice, Third Edition.* Colman RW, Hirsh J, Marder VJ & Salzman EW (eds). (J.B. Lippincott Company, Philadelphia, 1994).

[13] Takagi T & Doolittle RF. Amino acid sequence studies on the a chain of human fibrinogen. Location of four plasmin attack points and a covalent cross-linking site. *Biochemistry* **14**, 5149-5156 (1975).

[14] Takagi T & Doolittle RF. Amino acid sequence studies on plasmin-derived fragments of human fibrinogen: amino-terminal sequences of intermediate and terminal fragments. *Biochemistry* **14**, 940-946 (1975).

[15] Nomura S, Kashiwagi S, Ito H, Mimura Y & Nakamura K. Degradation of fibrinogen and fibrin by plasmin and nonplasmin proteases in the chronic subdural hematoma: evaluation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot. *Electrophoresis* **14**, 1318-1321 (1993).

[16] Ständker L et al. *In vivo* degradation of human fibrinogen Aa: detection of cleavage sites and release of antithrombotic peptides. *Biochem. Biophys. Res. Commun.* **215**, 896-902 (1995).

[17] Expert Protein Analysis System, ExPASy, SWISS-PROT database: www.expasy.ch/tools/protparam.html

[18] Kyte J & Doolittle RF. A simple method for displaying the hydropathic character of a protein. *J.Mol. Biol.* **157**, 105-132 (1982).

[19] L. Cammish, personal communication.

[20] M. Lütolf & J. Hubbell, unpublished results.

[21] Wellings DA & Atherton E. Standard Fmoc protocols. *Methods in Enzymology* **289**, Fields GB (ed.) (Academic Press, San Diego, CA, USA, 1997).

[22] Mieyal JJ, Starke DW, Gravina SA & Hocevar BA. Thioltransferase in human red blood cells: kinetics and equilibrium. *Biochemistry* **30**, 8883-8891 (1991).

[23] Johnson RS, Bowers M & Eaton Q. Preparation and characterization of *N*-(1-pyrenyl)iodoacetamide-labeled *Escherichia coli* RNA polymerase. *Biochemistry* **30**, 189-198 (1991).

[24] Yamamoto H, Kitsuki T, Nishida A, Asada K & Ohkawa K. Photoresponsive peptide and polypeptide systems. 13. photoinduced cross-linked gel and biodegradation properties of copoly(L-lysine) containing ε-7-coumaryloxyacetyl-L-lysine residues. *Macromolecules* **32**, 1055-1061 (1999).

[25] Expert Protein Analysis System, ExPASy, SWISS-PROT database: www.expasy.ch/swiss-shop

[26] Trowbridge CG, Krehbiel A & Laskowski, M, Jr. Substrate activation of trypsin. *Biochemistry* **2**, 843-850 (1963).

[27] Bechet J & Yon J. Mise en évidence d'un effet allostérique lors de l'hydrolyse du p-toluène sulfonyl-L-arginyl-méthyl ester par la trypsine. *Biochim. Biophys. Acta* **89**, 117-126 (1964).

[28] Anand S, Wu J & Diamond SL. Enzyme-mediated proteolysis of fibrous biopolymers: dissolution front movement in fibrin or collagen under conditions of diffusive or convective transport. *Biotechnol. Bioeng.* **48**, 89-107 (1995).

[29] Netzel-Arnett S, Fields G, Birkedal-Hansen H & Van Wart HE. Sequence specificities of human fibroblast and neutrophil collagenases. *J. Biol. Chem.* **266**, 6747-6755 (1991).

[30] Netzel-Arnett S et al. Comparative sequence specificities of human fibroblasts and neutrophil matrix metalloproteinases. *Matrix Metalloproteinases and Inhibitors.* Birkedal-

Hansen H, Werb Z, Welgus HG & Van Wart HE (eds). (Gustav Fisher Verlag, New York, 1992).

CHAPTER 4

IN VITRO CELL MIGRATION

A three-dimensional cell migration assay was developed and used to evaluate the design principles behind and the biomimetic character of a novel type of synthetic biomaterial which was formed *in situ*. Cell migration within synthetic, effectively non-porous, plasmin-degradable hydrogels, was observed. No significant cell migration was observed in synthetic materials insensitive to proteolytic enzymes involved in cell migration. Soluble inhibitors of plasmin and plasminogen activation inhibited cell migration in plasmin-sensitive materials, and addition of FGF-2 and PDGF-BB, growth factors that up-regulate plasminogen activation, increased cell migration. Changing the cross-link character of the gels also modulated cell migration rates. Cell adhesion and migration within the synthetic materials was RGD-dependent as demonstrated by the concentration-dependent effect of RGD on cell migration, and cell migration was inhibited when RDG instead of RGD was incorporated into the materials.

INTRODUCTION

Fibroblasts

Fibroblasts are the primary cell type of loose connective tissue, skin and tendon. They synthesize and deposit the majority of the ECM molecules and regulate extracellular matrix degradation and remodeling throughout the body. Collagen synthesis accounts for 5-10% of fibroblast total protein synthesis with fibroblasts generating different collagen types according to cell origin and site of deposition. Fibroblasts also produce fibronectin (1-3% of their protein synthesis), elastin, hyaluronic acid, and chondroitin sulfate, dermatan sulfate, and heparan sulfate proteoglycans. Matrix-degrading enzymes such as MMPs and serine proteases, as well as their inhibitors, are also generated by fibroblasts.[1, 2, 3, 4]

Because they are the principle architects responsible for building extracellular matrix, fibroblasts are important in wound healing for depositing repair tissue. Fibroblasts are assumed to be inherently motile, and they continuously explore their immediate environment unless hindered, e.g., by contact inhibition. They are, along with inflammatory cells and endothelial cells, the first cells to migrate into an injury site following clot formation. They are responsible for reorganization of the fibrin clot to form granulation tissue and later a collagenous connective tissue scar. However, they can also be responsible for such disease states as hypertrophic scars, post-inflammatory and post-operative adhesions, and organ, e.g., kidney, fibroses due to overproduction of connective tissue or to augmented deposition or cross-linking of ECM.[1] Hernia, a weakness of connective tissue, is another pathology for which fibroblasts are at least in part responsible.[2]

Fibroblasts are stromal cells (associated with bone marrow in origin) and are among the most plastic cell types in the body. Fibroblasts are dispersed throughout the body where

they vary from tissue to tissue as a result of differentiation during development.[1] Even in adults, different fibroblast phenotypes remain responsive to stimuli such as cytokines, growth factors, morphogens, and extracellular matrix which can regulate cell activity and differentiation. In specialized connective tissues, such as cartilage and bone, differentiated cells of the fibroblastic lineage, chondroblasts and osteoblasts respectively, assume unique phenotypes and deposit tissue-specific matrices.[2]

In culture, fibroblast morphologies vary as a function of the culture conditions, including substrate, medium, and cell density. In two dimensional culture on adhesive substrates such as tissue culture polystyrene and at low cell density, fibroblasts spread significantly, flattening to thicknesses of only a few microns and to lengths on the order of 50 μm.[5] When fibroblasts migrate on surfaces, large ruffled lamellipodia develop, predominantly at the leading edge; a large oval nucleus and cytoplasmic organelles reside near the center of the cell body and are followed by a stalk-like structure containing little cytoplasm at the rear of the cell. Stress fibers are observed in cultured fibroblasts but are not found in fibroblasts in tissues.[1, 3] At higher cell densities, near and at confluence, fibroblasts adopt a 10-20 μm high,[5] bipolar spindle shape for closer packing. While the proliferation of some cell types, such as endothelial cells, is contact inhibited, fibroblast proliferation is not. Fibroblasts can grow in multilayer culture until a saturation density is reached.[3] Their saturation density does not rely solely on cell-cell contact or contact inhibition, which predominantly tends to inhibit cell movement. The saturation density or number of monolayer equivalents ultimately observed is a function of cell origin and culture conditions; on average, five or six monolayer equivalents are formed.[2] In culture, fibroblasts exhibit aging and have limited replicating lifetimes.

In three-dimensional fibroblast culture, a long bipolar morphology is observed. The fibroblasts have more compact pseudopodia than in two dimensional culture, and the pseudopodia are found only at the ends of the cell. Also, the filopodia or spikes are relatively fine in three-dimensional culture.[2]

A specialized type of fibroblasts is the myofibroblast. Myofibroblasts are fibroblasts found in granulation tissue at the later stages of wound healing *in vivo*. Cultured fibroblasts can be morphologically and biochemically similar to myofibroblasts. Myofibroblasts also contain bundles of fibers like those in muscle cells which make the cells contractile, and sheets of cultured fibroblasts exhibit contractile forces similar to granulation tissue of the same cross-sectional area.[3] In spite of the similarities, myofibroblasts can be distinguished from fibroblasts in two- and three-dimensional cell culture due to the ruffled cell membrane and nucleus of myofibroblasts.[2]

Three-dimensional cell migration assays

To date, the majority of cell migration research has been performed using two-dimensional cell migration assays, that is to say using cells attached to surfaces, including coated surfaces and macroporous materials, rather than cells embedded within materials. However, with the most notable exceptions of endothelial and epithelial cells, most of the cells in the body exist within and migrate through three-dimensional materials rather than on surfaces, and matrices with pore sizes larger than cell processes are not observed. Therefore, three-dimensional cell culture systems where pore sizes are smaller than the length scale of cell processes, a few microns, are needed to model physiological cell behavior, such as migration and neurite outgrowth.

Physiologically, cells migrate by clearing their own paths. This is done by enzymes, plasmin or MMPs, localized in their active forms to the cell surface and the pericellular space. To evaluate the material design principles described in this work, primarily the enzymatic degradability of biomaterials and its necessity for cell migration within biomimetic materials, it was necessary to use a three-dimensional cell migration assay. Due to shortcomings of published assays, it was desirable to develop a new assay.

Published methods for studying cell migration in three dimensions include: Boyden chamber assays;[6] overlay[7] and sandwich methods; *in vitro* injury models;[8] and time lapse microscopy of cells in three-dimensional materials.[9] The Boyden chamber assay consists of a cell culture well separated top from bottom by a porous (e.g., 2-12 μm pore diameter) filter which is typically made of nitrocellulose or polycarbonate. Cells are seeded on top of the filter, and soluble compounds of interest are injected into the bottom chamber. The effect, e.g., induction of chemotaxis, of the injected compound is evaluated by the number of cells transversing the filter and counted on its bottom surface. One often coats the filter, e.g., with collagen or laminin, such that, depending on the coating conditions, cells must also migrate through a material of interest in order to get to the lower surface of the filter. However, the assay is rather complicated for evaluating synthetic materials as described herein because the filters are not inexpensive, uniform application of material can be difficult, and material swelling may lead to delamination or other discontinuities.

Overlay methods must overcome relatively strong adhesive forces between cells and tissue culture polystyrene in order to induce cell migration into an overlying material. Overlay and sandwich techniques can fail due to a poor union between materials at their interface. Cells are extremely sensitive to discontinuities and often will not migrate from one

fibrin matrix to another made in contact with the first. In injury models, a fibroblast culture is grown to confluence or supra-confluence and then scratched with a razor blade or needle. The cellular infiltration into the "injury" to "heal" it is quantified. Injury assays also suffer from material application problems similar to the Boyden chamber assay. Time lapse systems, where individual cells within a matrix are observed as a function of time, require specialized computerized microscopy techniques with lots of data storage space, special objectives and stages for depth profiling, and time consuming, relatively laborious data analysis methods.

Therefore, a relatively simple assay for cell migration in three dimensions was developed. Standard microscopy techniques could be used for evaluation. One could readily and continuously approximate where a cell had begun at time zero and therefore follow migration in the same culture samples as a function of time. One could easily compare materials or treatments with one another. The assay was inspired by some existing, published techniques: an agarose drop assay;[10, 11] a cell-coated fibrin fragment;[12] and three-dimensional explant cultures including a dorsal root ganglion assay for studying neurite extension in three dimensional materials, such as fibrin,[13] and an abdominal muscle fragment also cultured within fibrin.[12] In each of the assays, cells migrate out of and away from regions of high local cell concentration into regions of relatively low cell density driven by innate cellular motility under such conditions.

Fibrin drop assay

To avoid using tissue explants and to evaluate behavior of a single cell type, a cell-encapsulation technique was sought to test cell migration within biomaterials. A fibrin drop

assay was developed when it was observed that human fibroblasts did not migrate out of agarose beads into surrounding substrates such as fibrin or synthetic plasmin-degradable PEG-based hydrogels. This observation was not surprising since agarose is not degraded by fibroblasts, and the pores of agarose are too small for fibroblasts to penetrate agarose; fibroblasts within the agarose bead could not escape the agarose. Therefore a material which fibroblasts are known to penetrate, fibrin, was chosen as the material in which to prepare beads of fibroblasts.

When fibrin beads containing a high concentration of fibroblasts were cultured inside plasmin degradable PEG-based hydrogels described in this thesis, fibroblasts grew out of the beads in three dimensions with a spindle-shaped morphology. Behavioral and morphological similarities were observed in other materials, e.g., similarly cross-linked PEG-based materials which are degradable by MMPs instead of plasmin[14] and in photopolymerized protein-*co*-PEG hydrogels which are degradable by plasmin.[15] This three-dimensional assay was used in order to evaluate cellular behavior in different materials and under different culture conditions, for example in the presence of protease inhibitors or growth factors.

Advantages of the fibrin drop assay

The drop formation is gentle since fibrin formation endogenously occurs in contact with cells during blood clotting. The material preparation is simple; stock solutions can be prepared in advance and frozen and then need only to be thawed and diluted prior to use which involves only mixing and pipetting. Culture of the cell-clusters within three dimensions is easier than on a surface because the clusters cannot detach during culture or manipulation, such as medium change. Also, fibrin is degradable by cell activity; therefore

cells can migrate out of it and into the surrounding material if it is cell-permissive. This model of embedding clot-entrapped cells within a material of interest that is formed *in situ* results in an interface across which cells can migrate, something that is not trivial to accomplish.

Disadvantages of the fibrin drop assay

Depending on the extent of cross-linking, fibrin clots can retract after cross-linking; therefore one must be careful to prepare them in a consistent manner and allow the clots to retract before seeding them inside the material of interest. Also, during data collection and analysis, one should correct for any additional retraction that occurs during culture. This can be done by measuring the clot area at each time point investigated, instead of only using the clot area measured at time zero, in order to determine the area of the annulus of projected outgrowth and an area-averaged cell migration distance. Also, the assay requires the use of thrombin to initiate fibrin cross-linking. Thrombin is a proteolytic enzyme, and some cell-surface molecules are substrates for it.[16] Specifically, thrombin is a serine protease, and in this case the materials being evaluated are designed to be degraded by the serine protease plasmin.

FGF and PDGF

Fibroblast growth factors (16-17 kDa) are pleiotropic, heparin-binding growth factors broadly distributed in tissues. They are active in development and adult homeostasis. Acidic and basic forms are found and referred to as aFGF or FGF-1 and bFGF or FGF-2, respectively. Both lack a characteristic signal sequence for secretion, both are sequestered

within the extracellular matrix by binding to heparan sulfate, and both bind to the same cell-surface receptors, four of which have been identified. The FGF receptors include high affinity receptor tyrosine kinases and heparan sulfate proteoglycans with varying affinities.[17] Activation of FGF receptors by FGFs induces cell migration, DNA synthesis, proliferation, and differentiation *in vivo* during development, wound healing, and some pathologies. During migration and proliferation, FGFs affect cell attachment not by down-regulating integrin numbers or concentrations on the cell surface but rather by affecting integrin function and cytoskeletal organization.[18] Currently the intracellular signaling pathways activated by FGF activation of its receptors are actively being investigated for their influence on cell migration distinct from cell proliferation.[17]

Platelet-derived growth factors (PDGFs) are potent mitogens and chemoattractants for many cell types. They are dimeric proteins found mostly in platelets and bone, but also in other cell and tissue types, including endothelium, smooth muscle cells, and tumor cells.[19] They exist as A or B chain homodimers or AB heterodimers. PDGFs bind to receptor tyrosine kinases, PDGF-α receptor and -β receptor. The cellular response to receptor ligation varies according to the growth factor isoform-receptor isoform combination and depending on the cell type. The downstream effects of the PDGFs are to increase DNA expression and general protein synthesis, to stimulate cell division, and induce migration. Migration is induced as a result of activation of classical signaling pathways.[20]

Both FGF-2 and PDGF-BB have been shown to up-regulate uPA and uPA-R mRNA and protein levels without inducing PAI-1 secretion in human hepatic stellate cells. uPA-R occupancy was shown to also be increased by FGF-2 and PDGF-BB.[18] Hepatic stellate cells assume a myofibroblast-like phenotype following liver damage. In this form, they proliferate

and migrate into sites of tissue damage where they increase synthesis of ECM molecules. In a Boyden chamber assay with Matrigel-coated 8 μm filters, hepatic stellate cell migration was induced by FGF-2 and PDGF-BB. Each growth factor demonstrated its maximum effect at 10 ng/ml. The migration was inhibited by anti-uPA and anti-uPA-R antibodies.[18]

ε-aminocaproic acid

ε-aminocaproic acid (6-amino-hexanoic acid, EACA) is a lysine analogue used clinically as an antifibrinolytic agent. It reversibly occupies lysine binding sites in kringle domains on plasminogen[21] and at μM to low mM concentrations accelerates plasminogen activation to plasmin because of a conformational change. However, occupancy of the lysine binding sites prevents binding of plasminogen and plasmin to plasmin substrates, predominantly fibrin, and thus prevents proteolytic action of the plasmin. At high concentrations EACA may also act as a competitive inhibitor to reduce plasminogen activation[21, 22] It blocks plasminogen binding to cell surfaces thus reducing activation by uPA-uPAR mechanisms which account for a significant amount of plasminogen activation in tissues.[23] Therapeutically, EACA can be administered at relatively high doses (0.1 g/kg given intravenously, with plasma levels reaching 13 mg/100 mL or 1 mM). Tranexamic acid (trans-p-aminomethyl-cyclohexanecarboxylic acid, AMCA) is also used clinically, predominantly in Europe compared to EACA in the United States, and has 6-10-fold higher molar potency than EACA. Two other lysine analogues, p-aminomethylbenzoic acid (PAMBA) with 5-10-fold higher potency and 4-aminoethylbicyclo-[2.2.2]-octane-1-carboxylic acid (AMBOCA) with 100-fold higher potency than EACA, are less well studied than EACA and AMCA.[22]

Aprotinin

Aprotinin is the prototypic member of the kunin (**Kun**itz **in**hibitor) or aprotinin superfamily of proteins and is also known as pancreatic trypsin inhibitor, Kunitz, or by its trade name Trasylol. Each kunin domain of kunin proteins is comprised of approximately 58 amino acid residues with three disulfide bonds per domain.[24] Aprotinin contains just one kunin domain and is a relatively small protein (6500 Da) with high thermostability. It is isolated from bovine lung, parotid gland, and pancreas, and is a potent inhibitor of plasmin and other serine proteases, such as trypsin. Aprotinin forms stable electrostatic interactions with its target enzymes through interactions of its exposed Lys15 with acidic residues, e.g., Asp189 in trypsin. Aprotinin also has a high degree of surface structure complementarity with targets such as trypsin.[25] Aprotinin has been approved for clinical use in some countries, including use in commercial fibrin glues available in the United States since 1998.[26]

Aphidicolin

Aphidicolin (338.5 Da) is a selective inhibitor of DNA polymerase-α without affecting rates of protein synthesis.[27] It is isolated from the fungus *Cephalosporium aphidicola* and is a potent antiviral, antibiotic, and antimitotic agent. Use of aphidicolin (20 μg/mL) is recommended in agarose drop assays in order to eliminate effects of cell proliferation on cell migration.[11]

MATERIALS AND METHODS

Formation of fibrin drops with human dermal fibroblasts

Fibrinogen (low plasminogen content; Fluka, Buchs, Switzerland) was dissolved at 35 mg/3 mL in water and dialyzed against 4 L of Tris buffered saline (TBS: 28 mM Tris[hydroxymethyl]aminomethane hydrochloride (4.36 g/L), 5 mM Tris[hydroxymethyl]aminomethane (0.64 g/L), 137 mM NaCl (8.0 g/L), 2.7 mM KCl (0.2 g/L), pH 7.4 at 25°C) overnight at room temperature. The concentration of fibrinogen was then determined using its extinction coefficient of 1.55 at 280 nm.[28] Aliquots of fibrinogen were frozen until use and were thawed only once prior to use.

Human foreskin fibroblasts (Clonetics, Walkersville, MD, USA) were grown on tissue culture polystyrene until almost confluent and were then passaged with trypsin-EDTA solution (Gibco/Life Technologies AG, Basel, Switzerland). After neutralization of the trypsin with 10% fetal bovine serum-containing medium (Gibco), the cells were counted, then centrifuged (500x g, 5 min) and the supernatant removed. Just prior to use, thawed fibrinogen solution was diluted to 2 mg/mL with phosphate buffered saline (PBS: 2.7 mM KCl (0.2 g/L), 1.5 mM KH_2PO_4 (0.2 g/L), 137 mM NaCl (8.0 g/L), 8 mM $Na_2HPO_4 \cdot 7H_2O$ (2.16 g/L), pH 7.4). Thrombin (human, Sigma T6884, St. Louis, MO, USA, 20 U/mL in PBS) was added to fibrinogen solution to give 0.25 unit thrombin/ml fibrinogen solution. Cells were resuspended at 3.3 x 10^7 cells/mL in fibrinogen solution. Without delay, 1.5 - 2 μL droplets of the cell suspension solution were transferred to glass cover slips which were previously silylated (SigmaCote, Sigma) and sterilized by 70% ethanol. The fibrin drops were then allowed to polymerize for 15-20 minutes at 37° C, 5% CO2, and ≥95% relative humidity. Each fibrin clot contained approximately 60,000 cells.

Material formation and cell encapsulation

PEG-divinylsulfone (PEG-diVS) was synthesized according to Chapter 2. Peptides described in Table 4. 1 were synthesized according to Chapter 3.

Table 4. 1 Peptide sequences used to form synthetic hydrogels by conjugate addition

Sequence	Description	Abbreviation
AcG**C**GYG<u>RGD</u>SPG-NH2	integrin ligand containing RGD	RGD
AcG**C**GYGR<u>DG</u>SPG-NH2	non-integrin binding control peptide	RDG
G**C**YKNR**C**YKNR**C**G-NH2	tri-cysteine plasmin-sensitive cross-linker	C3
G**C**Y-*D*LysN-*D*Arg**C**Y-*D*LysN-*D*Arg**C**G-NH2	tri-cysteine plasmin-insensitive cross-linker	*D*C3
G**C**YKNR**C**YKNR**C**YKNR**C**G-NH2	tetra-cysteine plasmin-sensitive cross-linker	C4

To form materials, 110 μl of HEPES buffered saline (HEPES BS: 50 mM N-[2-Hydroxyethyl]piperazine-N'-[4-butanesulfonic acid] (13.02 g/L), 137 mM NaCl (8.0 g/L), pH 7.6 ± 0.2) were added to 15.8 mg PEG-diVS. Following dissolution, the entire volume of PEG-divinylsulfone (approximately 120 μl) was used to dissolve 1.2 mg RGD or <u>RDG</u> peptide. After mixing, this solution was allowed to stand for approximately 2 min before adding it to 4.3 mg tri-cysteine cross-linking peptide or 4.6 mg tetra-cysteine cross-linking peptide and mixing. Four well-spaced 25 μl aliquots of gel precursor were pipetted onto sterile, silylated (SigmaCote) glass slides (7.5 cm x 2.5 cm). With forceps, fibrin drops of fibroblasts were transferred such that there was one drop in the center of each 25 μl aliquot of precursor solution. A rectangular Teflon sheet (approximately 2.5 cm x 1 cm) of 700 μm thickness was placed at each end of the slide without touching the samples. A second sterile silylated glass slide was clamped on top of lower slide with samples and Teflon spacers using

ordinary binder clamps (Figure 4. 1). Materials gelled in less than 10 minutes but were cured for 1 hour at 37° C, 5% CO2, and ≥ 95% relative humidity in a standard cell culture incubator.

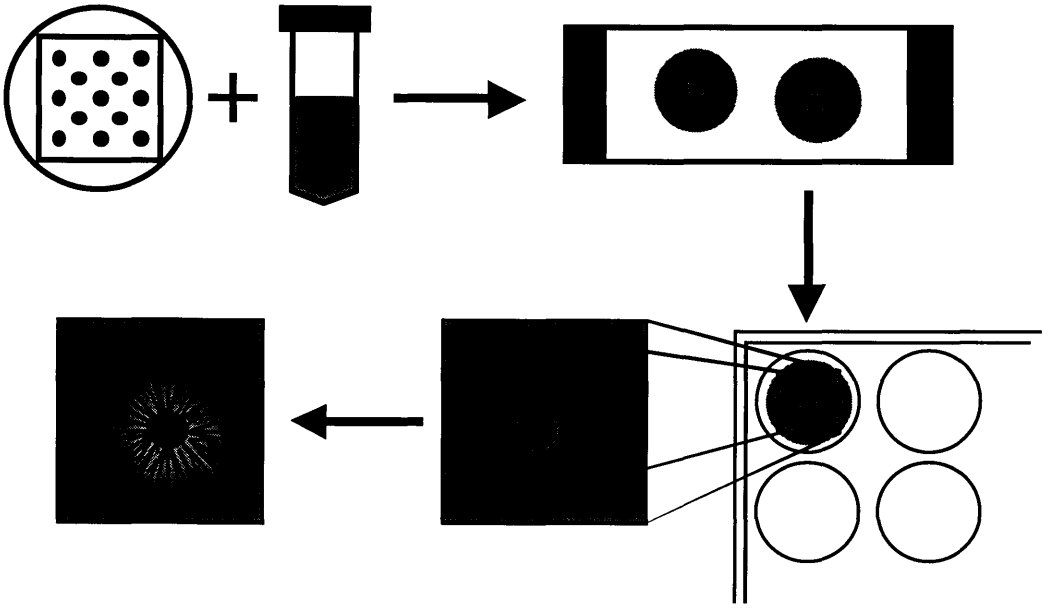

Figure 4. 1 Cell encapsulation and three-dimensional culture technique

RGD concentration series

To vary the RGD concentration within the materials without affecting the cross-link properties of the materials, N-acetyl-L-cysteine (Calbiochem-Novabiochem, Laüfelfingen, Switzerland) was incorporated in place of "missing" RGD when compared to the maximum RGD concentration tested and where 1 of 10 vinylsulfones was modified. In these studies, RGD peptide and cysteine were first separately dissolved and then added in the appropriate ratios (Table 4. 2) to the PEG-diVS.

Table 4. 2 RGD concentrations during gel formation and after swelling

	RGD (1.2 mg/110 μl)	AcCys (1.67 mg/ml)	[RGD] in precursor solution	[RGD] in gel after swelling*
100%	110 μl	-	10 mM	4 mM
50%	55 μl	55 μl	5 mM	2 mM
10%	11 μl	99 μl	1 mM	400 μM
1%	1.1 μl	108.9 μl	100 μM	40 μM

*Based on an averaged volumetric swelling factor of 2.5±0.1

Cell culture in three dimensions

After curing, gels containing fibroblasts were transferred with a sterile spatula to 12-well cell culture plates where they were free-floating with one gel and 1 ml of medium per well. Normal cell culture medium was Dulbecco's modified Eagle's medium (DMEM) without phenol red and containing 10% heat inactivated fetal bovine serum and 1% antibiotic and antimycotic solution (Gibco/Lifetechnologies). Serum was the source of plasminogen in all experiments (Murphy et al.).[29] Unless otherwise noted, medium changes were performed at 1-2 hr, 24 hr, 4 d, 7 d, and then twice per week. The times of medium changes were standardized due to their effect on cell migration rates. Medium changes were complete unless gels had become very swollen and soft due to degradation; in these cases, 50% medium changes were made instead.

Culture in the presence of exogenous factors

When exogenous factors were used, they were added first at the 24 hr medium change and then during all later changes unless noted otherwise. Factors were not added before 24 hr in order to avoid interference with the ability of the cells to migrate out of the fibrin clot, which is plasmin dependent. Aprotinin (Sigma) was used at a final concentration of 0.23

TIU/mL (6 μM). ε-aminocaproic acid (Sigma) was dissolved just before use to 200 mM[30] in culture medium and filter sterilized with low protein binding filters. FGF-2 and PDGF-BB (recombinant human, PeproTech, London, England) were stored in frozen aliquots according to the manufacturer's instructions and used at a final concentration of 10 ng/mL. Aphidicolin (Fluka) was stored at –20 °C in dimethylsulfoxide (DMSO) and used at a final concentration of 2 μg/mL. Control experiments with DMSO alone at this concentration (1 μL/mL) did not effect cell migration.

Cell viability staining

Fluorescein diacetate (Fluka) was dissolved at 5 mg/mL in acetone and stored at 4 °C in the dark. Cell cultures were twice washed with PBS and then 2 μL fluorescein diacetate solution was added to 1 mL PBS per well. After 2-5 minutes, cultures were again washed with PBS and viewed using fluorescence microscopy ($\lambda_{ex} \approx 490$ nm, $\lambda_{em} \approx 520$ nm). Ethidium bromide stock solution (2.5 mM) was prepared in 1 part DMSO: 4 parts water. Simultaneous with the addition and removal of fluorescein diacetate, 1-2 μL of ethidium bromide solution was applied. Ethidium staining was viewed using fluorescence microscopy ($\lambda_{ex} \approx 518$ nm, $\lambda_{em} \approx 605$ nm). After use, ethidium bromide-containing solutions were neutralized with hydrogen peroxide solution, and cell cultures were terminated.

Live cell nuclear staining

Hoechst 33342 (Sigma) was dissolved at 10 mg/mL in water. Cell cultures were twice washed with PBS and then 0.5 μl Hoechst solution per mL PBS was added to culture

samples. After 15 min incubation, culture samples were twice washed with PBS, and then the culture medium was replaced. Samples were viewed using fluorescence microscopy ($\lambda_{ex} \approx 350$ nm, $\lambda_{em} \approx 460$ nm).

Microscopy and image analysis

Images were acquired with an Axioscope using a 2.5x Plan or 4.0x Achroplan objective (Zeiss, Zürich, Switzerland) unless otherwise indicated. Images were digitized with a Shimatsu chilled color 3-chip CCD camera, Matrox Meteor PCI frame grabber (Matrox Electronic Systems, Dorval, Quebec, Canada), and Leica QWin image analysis software (Leica, Zürich, Switzerland). Image analysis was performed using Leica QWin. The projected area of the halo of three-dimensional outgrowth from fibrin clots into synthetic hydrogels was measured in a focal plane at the approximate center of a clot, and an area-averaged cell migration distance was calculated as the radius of an annulus.[13]

Statistics

Values are given as means ± average deviations. P values were calculated using a two-tailed, nonpaired Student t test.

RESULTS

Synthetic hydrogels were transformed from liquid precursors to viscoelastic solids in direct contact with clotted cells. Gels were made with tri-cysteine plasmin-sensitive cross-linkers and contained 4 mM RGD unless otherwise indicated. Since the gels were colorless

and transparent, cells cultured inside them could be viewed using standard light microscopic techniques, such as differential interference contrast microscopy, phase contrast microscopy, and fluorescence microscopy. Human fibroblasts were used because they are easy to culture, inherently motile, and important in wound healing and remodeling of extracellular matrices by plasmin-dependent mechanisms.

Cell migration within adhesive, plasmin-degradable hydrogels

Human fibroblasts migrated out of fibrin clots into synthetic plasmin-degradable hydrogels containing RGD peptides that were bound covalently and displayed in a dangling fashion throughout the gels. Cells extended into the synthetic materials in all dimensions around the clot. In doing so, cells assumed a long spindle-shaped morphology (Figure 4. 2). Such three-dimensional migration could be readily distinguished from cells which sometimes, when incompletely surrounded by hydrogel, grew out as two-dimensional monolayers of flattened cells on the surfaces of the hydrogels. In three dimensions, fibroblasts not only extended processes into the degradable hydrogel material, but also whole cells migrated into the material as observed by live cell nuclear staining with Hoechst 33342. Oval nuclei were observed in the halo of outgrowth in the hydrogel well away from the fibrin clot, e.g., as much as 800 μm at 7 d (Figure 4. 3).

Live cells, distinguishable from nonviable cells by staining with fluorescein diacetate and not with ethidium bromide, could be observed within the synthetic materials for at least as long as six weeks (Fig. 4. 4). Counting of cells to determine a percent viability was not possible due to the high local concentration of cells within the materials. Culture times as

long as six weeks were only possible in materials with modified cross-link character or in the presence of a plasmin inhibitor such as ε-aminocaproic acid.

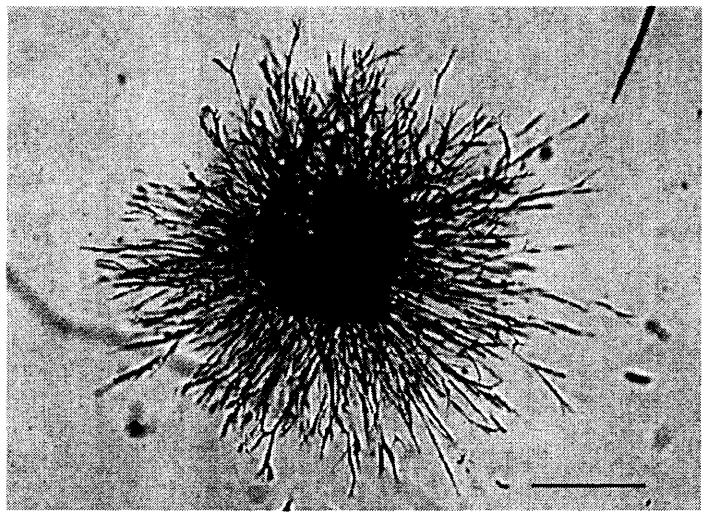

Figure 4. 2 Three-dimensional fibroblast outgrowth from a fibrin clot into a synthetic hydrogel formed *in situ*

Fibroblasts assume a spindle-shaped morphology as they migrate out of a 2 μL fibrin clot into a synthetic hydrogel containing plasmin-degradable cross-links and cell-adhesive RGD ligands. Day 7. Scale bar = 500 μm. Differential interference contrast microscopy.

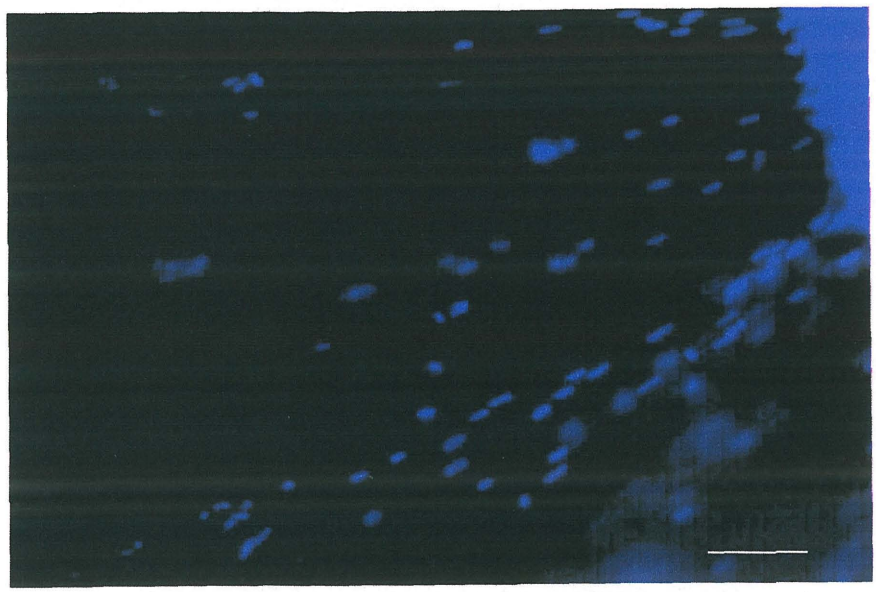

Figure 4. 3 Live cell nuclear staining of fibroblasts within a synthetic hydrogel

Staining of cell nuclei with Hoechst 33342 shows migration of whole cells out of a fibrin clot (dense blue staining in the upper right-hand corner) and into a synthetic plasmin-degradable hydrogel. Day 7. Scale bar = 100 μm. Fluorescence microscopy.

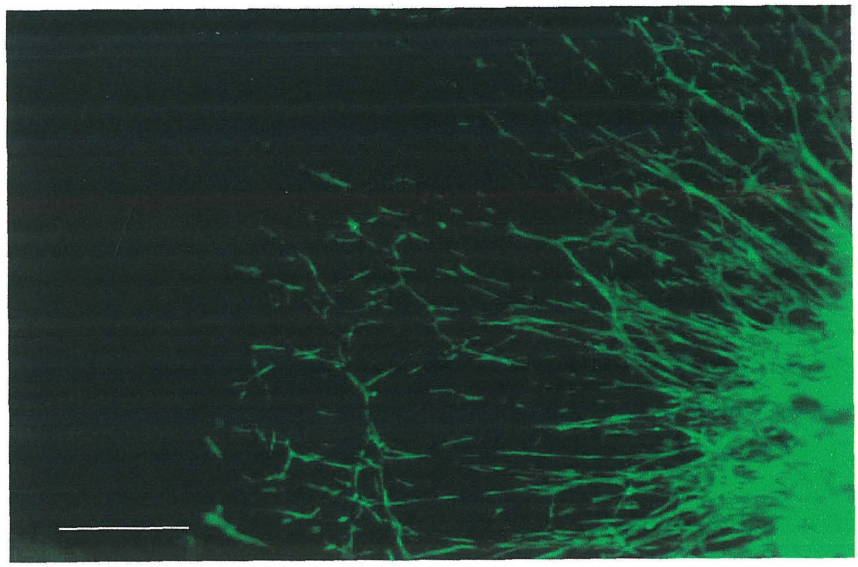

Figure 4. 4 Live cell staining at 6 wk within a synthetic plasmin-degradable hydrogel

Staining with fluorescein diacetate shows viable cells in a synthetic hydrogel after 6 wk in culture. The number of nonviable cells (not shown) was significantly lower and confined to the dense fibrin clot of cells (bright green stain in the lower right-hand corner). Scale bar = 150 μm. Fluorescence microscopy.

When fibroblasts were cultured without ε-aminocaproic acid within degradable materials containing 4 mM RGD, measurable migration started between 2 d and 4 d and persisted for 11-14 d. Beyond this duration, material degradation was so significant as to lead to mechanical instability of the materials, partial detachment and retraction of the fibroblasts, and termination of the experiments. During the peak migration period from 2-11 or 14 d, migration rates averaged 77 ± 13 μm/d. The average migration rate for the whole period, from the start of the three-dimensional culture until its collapse, and including the approximate 2 d lag where no migration was observed, was 67 ± 18 μm/d.

Comparing two sources of PEG-divinylsulfone

Cell culture experiments were initiated with a commercial source of PEG-diVS (Shearwater Polymers, Huntsville, AL, USA) that later became unavailable. Therefore, PEG-diVS was synthesized according to Chapter 2. Before cell culture experiments were continued, the fibroblast behavior within materials made separately from the two different sources of PEG-diVS was compared. No differences were observed (Figure 4. 5).

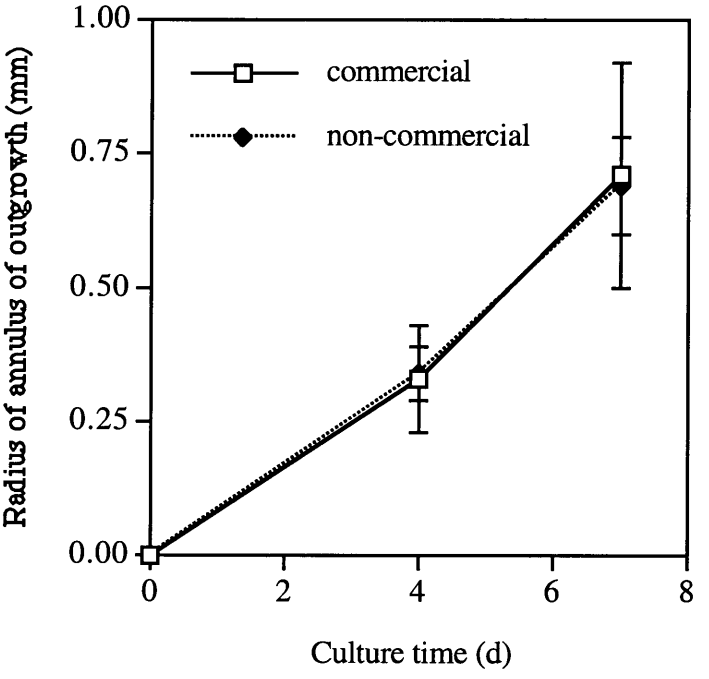

Figure 4. 5 Cell migration within materials prepared with PEG-diVS from two sources

Altering the biomimetic character of the synthetic materials

RGD concentration series

Altering the concentration of RGD within C3-containing materials from 40 μM to 4 mM affected the rate of cell migration in a concentration dependent manner (Figure 4. 6). At higher RGD concentrations, cell migration was initiated earlier and occurred more rapidly than in materials with lower RGD concentrations. Also, the maximum distance reached by the fibroblasts was highest in materials containing 4 mM RGD and decreased in a concentration dependent manner. This was observed even when cultures containing lower RGD concentrations were cultured for longer time periods (24 d) to account for delayed outgrowth and slower migration rates.

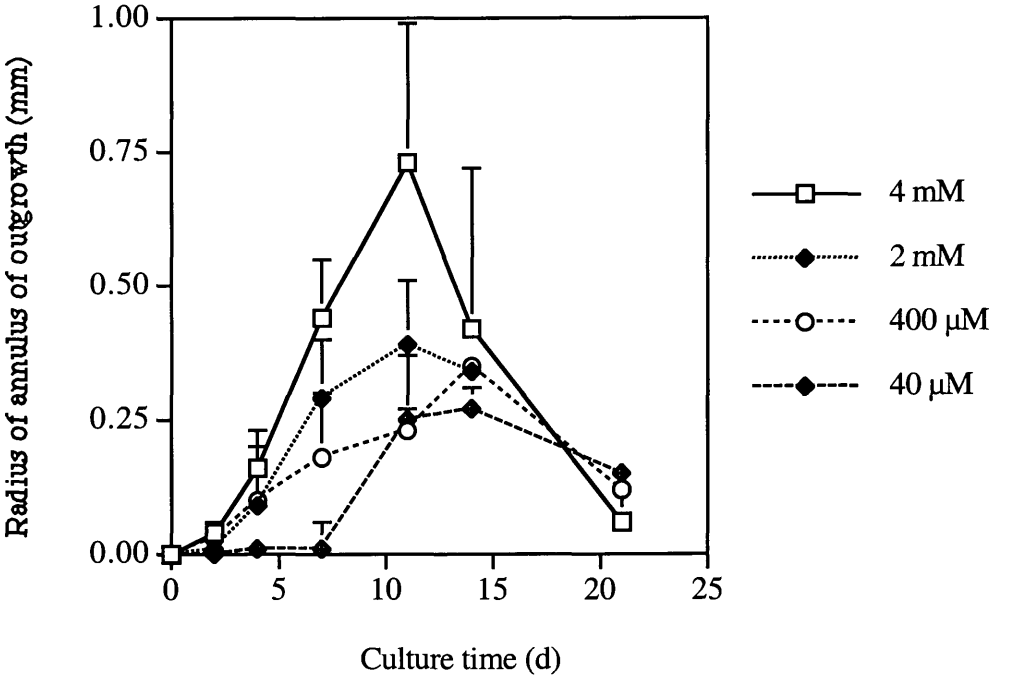

Figure 4. 6 Density of cell adhesion ligand affects rates of cell migration in three dimensions

RDG

When an RDG[31] ligand was incorporated (4 mM) instead of the RGD ligand, significantly less fibroblast outgrowth and migration was observed (74% ± 6% inhibition averaged for all time points to 14 d; $p < 0.01$).

Plasmin insensitive materials

When a *D*C3 was used as the cross-linker, no significant cell migration was observed for as long as three weeks when the experiment was terminated due to loss of cell viability. At 4 d and 7 d, a few short (≤4 μm) cell processes could be seen protruding from the fibrin into the synthetic material; these cells were probably observed due to retraction of the fibrin clot and the slower retraction of cells into the clot. Cells were not observed beyond the

original radius of the fibrin clot. This inability of fibroblasts to penetrate materials cross-linked with the *D*C3 corresponded with the plasmin-insensitivity of *D*C3 in solution phase kinetic studies and the inability of plasmin to degrade materials cross-linked with the *D*C3 (Chapter 3).

Influence of network structure

When a tetra-cysteine peptide (C4) was used as the degradable cross-linker, slower cell migration was observed in comparison to materials containing the degradable tri-cysteine peptide (C3) (Figure 4. 7). Also, longer culture times were possible in the C4 materials, and cells were viable for at least as long as 6 wk (Figure 4. 4). Slower bulk degradation of the materials and slower cell-mediated degradation of the materials was therefore observed in the C4 materials. C4 materials contained 50% more plasmin degradable sites per swollen gel volume and statistically required cleavage of 70% more plasmin degradable bonds to break each cross-link. Maximal migration distances obtained in the C4 materials were at least as great as in the C3 materials when the experiment was terminated. At that point, no excessive bulk degradation and no cell retraction had yet been observed in the C4 materials.

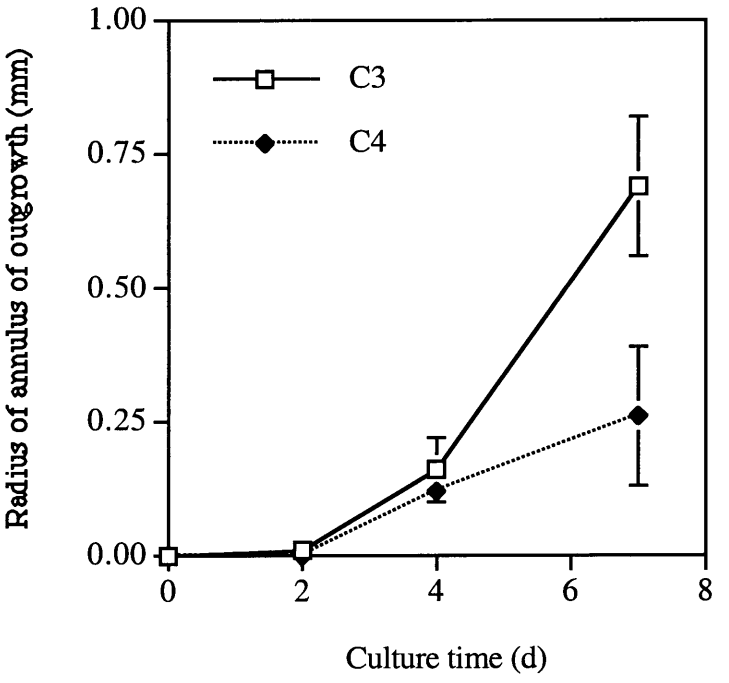

Figure 4. 7 Network cross-link structure affects cell migration rates

Manipulation of the fibrinolysis cascade

In the presence of exogenous inhibitors of plasmin activity, cell migration was inhibited. Aprotinin inhibited fibroblast migration by 19% ± 6% as averaged at 4 d and 7 d ($p < 0.5$). EACA inhibited fibroblast migration 80% ± 13% at 4 d and 7 d ($p < 0.05$) (Figure 4. 8). Aprotinin and ε-aminocaproic acid partially reduced plasmin activity and thus permitted highly localized degradation near the cell surface while minimizing bulk degradation ahead of the cell-material interface. As a result, cultures containing aprotinin and EACA could be maintained longer than cultures without. Conversely, addition of FGF-2 or PDGF-BB increased the rate of cell migration within the synthetic plasmin-sensitive materials (Figure 4. 8). Controls and growth factor-treated samples contained aphidicolin to prevent cell proliferation.

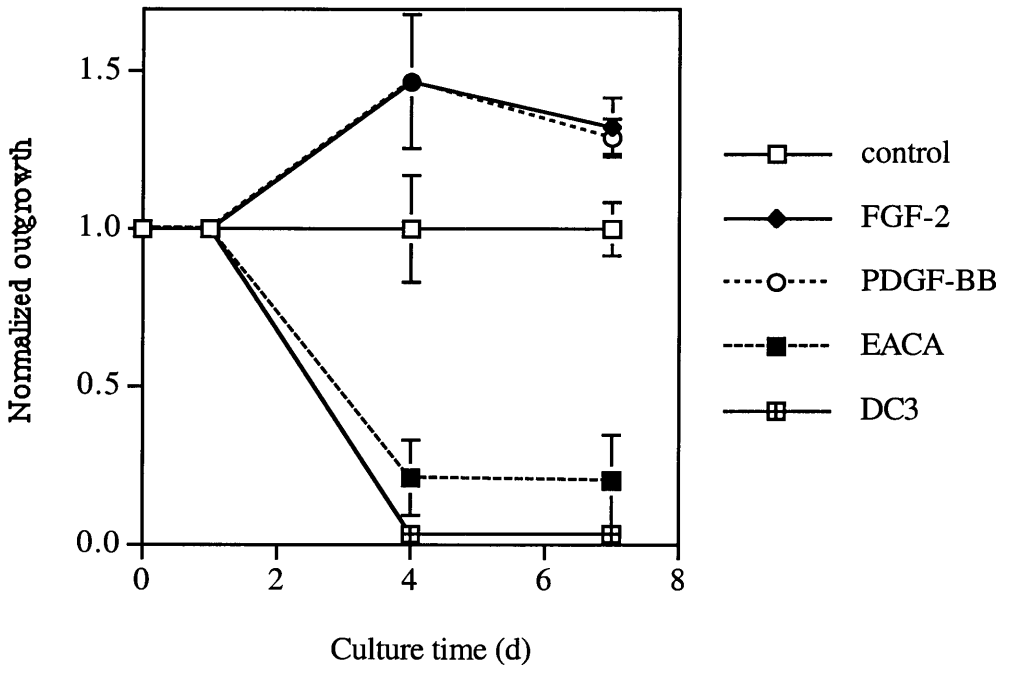

Figure 4. 8 Cell migration rates in synthetic materials are modulated by modulators of the fibrinolytic cascade

DISCUSSION

A novel three-dimensional cell migration assay was developed using droplets of fibrin-clotted cells within materials. The assay can be used as long as the materials of interest can be formed *in situ*. The droplet formation is gentle, and droplet preparation is simple. The assay was used to evaluate the design principles behind and the biomimetic character of a novel type of synthetic hydrogel which can be formed *in situ*.

One potential disadvantage of the assay is the exposure of cells to thrombin during fibrin drop formation. Thrombin is a serine protease, which may have implications specific to the materials evaluated in this work since they are designed to be degraded by another serine protease, plasmin. However, thrombin is not expected to degrade the substrate used in this work based on its preference for Arg- with aliphatic residues, specifically Pro, Ala, Ile,

and Leu, in the P2 position and Gly, Ser, and aliphatic residues in the P1' position. While substrate degradability by plasmin and collagenase were characterized, a recommendation for future work would be to characterize substrate susceptibility to a larger assortment of enzymes relevant to wound healing and used in *in vitro* assays.

There are also some general concerns about using thrombin in cell migration assays. Some cell surface molecules, including a set of protease-activated, G-protein-coupled receptors, are substrates for the enzyme. Signaling through the receptors helps regulate fibrinolysis, cell growth, and cell migration. Therefore, at least for an initial culture time, one is observing cell responses to materials from cells that have been treated with thrombin, a regulator of cell behavior. To minimize the effect of thrombin on the cells by reducing the duration of thrombin exposure, one could wash the fibrin drops, e.g. by soaking them in excess buffer, medium, or protease inhibitor solution, before initiating culture within the materials of interest. Alternatively, one could use a minimum or physiological concentration thrombin during fibrin drop preparation. Since thrombin is present physiologically during hemostatic responses that proceed wound repair, its affect may not be entirely detrimental to the evaluation of potential materials for wound healing applications but should not be ignored.

Similarly, one should consider the effect of trypsin on the cell migration assay. Trypsin, another serine protease, was used to passage the cells prior to encapsulation. While excess trypsin was neutralized with serum-containing medium and removed from the cells prior to encapsulation, trypsin had time during passaging to cleave cell-surface molecules. Cell-surface molecules affected by trypsin include integrins, growth factor receptors, and tPA and uPA receptors.[32] This may account, at least in part, for the approximate two day lag time

during which no cell migration out of the fibrin drops and into plasmin-degradable materials was observed. The cells may have had to renew cell surface molecules, such as integrins and plasminogen activator receptors, in order to initiate material degradation and cell migration. In fibrin alone, trypsinized fibroblasts demonstrated similar lag times for extension as well as proliferation.[33] An alternative to trypsinization would be to passage subconfluent cells with ethylenediaminetetraacetic acid and ethyleneglycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid solution (such as Gibco's Versene) to chelate divalent cations required for cell attachment, e.g., by integrins.

Cell migration was observed within synthetic plasmin-degradable materials formed *in situ*. No significant cell migration was observed in synthetic materials insensitive to proteolytic enzymes involved in cell migration. Cell migration in plasmin-sensitive materials could be inhibited by soluble inhibitors of plasmin while migration could be increased by up-regulating the plasminogen activation pathway with growth factors. Changing the cross-link character of the gels also modulated cell migration rates. Cell adhesion and migration within the synthetic materials was RGD-dependent as demonstrated by the concentration-dependent effect of RGD on cell migration and the inhibition of cell migration when RDG instead of RGD was incorporated into the materials. These observations indicate that both components of the material design, the enzymatic degradability and the specifically mediated cell adhesion, in this case integrin-mediated by RGD ligands, are required for cell extension and migration within three-dimensional matrices such as synthetic extracellular matrices.

In degradable tri-cysteine peptide cross-linked materials, rates of cell migration were maximally on the order of 100 μm/day. This rate is approximately 10% of that observed in a biological matrix such as collagen.[34] Therefore, while the synthetic hydrogels described

herein contain the minimum biomimetic character, cell adhesion and enzymatic degradability, required for cell-migration in three-dimensional matrices, they do not support migration rates of the same order of magnitude of naturally occurring biomaterials. There may be a number of reasons for the lower migration rates in the synthetic materials. (1) The synthetic materials contained RGD as the only ligand for cell attachment. Therefore, they may not present the optimal ligand or combination of ligands, or they may not present the optimal concentration of ligand. (2) No single protease is solely responsible for matrix turnover. Therefore, relying on one only may insufficiently mimic physiological behavior and fail to provide optimum or adequate degradation rates. (3) Substrate mechanical properties have been demonstrated to affect cell motility,[35, 36] and mechanical properties, a parameter that was not varied in these studies, may not be optimal. Mechanical properties were held as constant as possible in order to evaluate other variables, such as ligand concentration. However, in the case of the C4 materials, the mechanical properties of the materials differed from those of the C3 materials and can not be eliminated as a source of the differences observed between the two. (4) Also the degradability of the materials, as determined by the enzyme-substrate kinetics and the network structure, is not yet optimized for maximal rates of cell migration.

In degradable tri-cysteine peptide cross-linked materials, rates of bulk degradation were so significant as to lead to premature loss of mechanical stability of the materials. This was probably due to diffusion of active plasmin away from the cell surface, where it was predominantly activated from plasminogen in the medium, and into the material where it degraded the plasmin-sensitive cross-links without regulation. In physiological matrices, bulk degradation in advance of the cell-matrix interface is not observed. On the contrary,

matrix degradation and remodeling is a highly controlled process involving soluble and matrix-bound protease inhibitors, such as α2-plasmin inhibitor. α2-plasmin inhibitor is cross-linked to fibrin by factor XIIIa during clot formation where it helps to limit plasmin activity to the pericellular space.

One should be able to reduce the rate of bulk degradation in the synthetic materials by design in a number of ways. (1) Addition of a plasmin-inhibitor, either in soluble or bound form, would reduce the rate of bulk degradation, and this was observed by longer culture times before the loss of mechanical properties due to the presence of soluble aprotinin and EACA. (2) A change in network structure can reduce the rate of bulk degradation. In the tri-cysteine peptide cross-linked materials, only one bond per cross-link is required to break the cross-link. In the tetra-cysteine peptide cross-linked materials, statistically breaking of more than one bond is required to break a cross-link. This change in network structure slowed the rate of bulk degradation such that no significant loss of mechanical properties was observed in 6 wk compared to 11-14 d with the tri-cysteine peptide cross-linked gels. One can also change the network architecture not only by increasing the functionality of the cross-linking peptide but also by increasing the functionality of the copolymer, in this case the PEG, also effectively diluting the concentration of degradation sites. Slower degradation rates are observed in gels with such a geometry and collagenase degradation sites.[14] (3) Changing the enzyme substrate (Chapter 3) would also affect the rates of both local and bulk degradation. It is still being determined whether the kinetics of material degradation are controlled more by material architecture or enzyme-substrate kinetics (K_m and k_{cat}), providing an interesting area of study.[37]

The rate of degradation in the synthetic materials, as in fibrin, may be auto-accelerated by generation of carboxy-terminal lysines within the material upon partial degradation. Plasmin(ogen) binds to these zwitterionic lysines better than to non-zwitterionic lysines in the material backbone, thus increasing plasmin activity in an auto-catalytic mechanism. One way to solve this problem, distinct from changing K_m or network structure, would be to use an arginine-containing substrate for plasmin or combine lysine-containing and arginine-containing substrate cross-linkers to obtain an optimal relative concentration of the two.

In the RGD concentration range tested, a biphasic response of migration rate to ligand concentration was not observed, and saturation of RGD concentration was also not observed. Evaluation of higher ligand concentrations would have required a change in cross-linking properties, modification of more than 1/10 vinylsulfones, the ratio maintained throughout this work, and possibly a change in network structure, including precursor functionality. A biphasic response of migration rate to ligand concentration and cell-substrate adhesiveness has been modeled and observed *in vitro* in two dimensions[38, 39] and in three dimensions.[40] When ligand density or cell-substrate adhesiveness is low, cell migration is slow due to poor traction. When ligand density is high, migration is again slow due to the stickiness of the substrate; cells must overcome the avidity or cooperativity of numerous binding events which requires a relatively large number of dissociation events to occur simultaneously in order for a cell to move. Between these two extremes is an optimal cell-substrate adhesiveness. The implication is that in applications where cell migration is desired for functional wound healing, e.g., re-endothelializaton of a vascular graft or nerve regeneration over a long gap,

an optimum of cell-substrate adhesiveness, e.g., ligand identity and concentration, must be found.

In three dimensions, similar biphasic responses of the rate and extent of migration to degradability are also hypothesized. When degradation is slow, the physical barrier of the material prevents cell migration. Rapid material degradation limits cell migration by prematurely removing the foothold for cells. And again, an optimum between the two extremes is hypothesized. Evaluating the hypothesis is complicated by the presence of both local and bulk degradation; therefore, degradation should be limited to pericellular proteolysis only.

Coupled to cell degradation of biological matrices is deposition of new matrix molecules. As cells migrate into the degradable synthetic materials described here, an opaque, white material can be observed between the fibrin clot and the leading edge of the cell front, and its density increases with time. When unregulated enzymatic degradation causes loss of cell traction at the leading edge of the cell front, the cells retract. However, they do not retract completely to the fibrin clot; they retract to the borders of the opaque deposits. The deposits have yet to be analyzed for content, but it is hypothesized that they contain collagen, fibronectin, and glycosaminoglycans. Frozen samples will be cryo-sectioned and immunologically stained for extracellular matrix molecules. What the staining may not resolve is whether the molecules are sufficiently organized and cross-linked by cell-mediated processes or instead precipitated, e.g., due to the presence of PEG, the major component of the synthetic hydrogels, which is known to drive proteins out of solution.

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REFERENCES

[1] Sappino AP, Schürch W & Gabbiani G. Differentiation repertoire of fibroblastic cells: expression of cytoskeletal proteins as marker of phenotypic modulations. *Lab. Invest.* **63**, 144-161 (1990).

[2] Kulonen E & Pikkarainen J. *Biology of fibroblast.* (Academic Press, New York, 1973).

[3] Gabbiani G & Rungger-Bändle E. The fibroblast. *Handbook of inflammation: tissue repair and regeneration.* Glynn LE (ed.) (Amsterdam, Elsevier/North-Holland Biomedical Press, 1981).

[4] Holbrook KA & Smith LT. Morphology of connective tissue: structure of the skin and tendon. *Connective Tissue and Its Heritable Disorders: Molecular, Genetic, and Medical Aspects.* Royce PM & Steinmann B (eds). (Wiley-Liss, Inc., New York, 1993).

[5] Folkman J & Moscona A. Role of cell shape in growth control. *Nature* **273**, 345-349 (1978).

[6] Boyden S. The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J. Exp. Med.***115**, 453-466 (1962).

[7] Brown LF, Lanir N, McDonagh J, Tognazzi K, Dvorak AM, & Dvorak HF. Fibroblast migration in fibrin gel matrices. *Amer. J. Pathol.* **142**, 273-283 (1993).

[8] Auerbach R, Auerbach W & Polakowski I. Assays for angiogenesis: a review. *Pharmac. Ther.* **51**, 1-11 (1991).

[9] Dickinson RB, McCarthy JB & Tranquillo RT. Quantitative characterization of cell invasion in vitro: formulation and validation of a mathematical model of the collagen gel invasion assay. *Annal. Biomed. Eng.* **21**, 679-687 (1993).

[10] Varani J, Orr W, & Ward PA. A comparison of the migration patterns of normal and malignant cells in two assay systems. *Amer. J. Pathol.* **90**, 159-172 (1978).

[11] Frost EE, Milner R, & ffrench-Constant C. Migration assays for oligodendrocyte precursor cells. *Methods in Molecular Biology* ***139***, *Extracellular Matrix Protocols*. Streuli CH & Grant ME (eds) (Humana Press, Totowa, NJ, 2000).

[12] Hiraoka N, Allen E, Apel IJ, Gyetko MR & Weiss SJ. Matrix metalloproteinases regulate neovascularization by acting as pericellular fibrinolysins. *Cell* **95**, 365-377 (1998).

[13] Herbert CB, Bittner GD & Hubbell JA. Effects of fibrinolysis on neurite growth from dorsal root ganglia cultured in two- and three-dimensional fibrin gels. *J. Compar. Neurol.* **365**, 380-391 (1996).

[14] M. Lütolf & J. Hubbell, unpublished results.

[15] S. Halstenberg & J. Hubbell, unpublished results.

[16] Coughlin SR. Thrombin signaling and protease-activated receptors. *Nature* **407**, 258-264 (2000).

[17] Boilly B, Vercoutter-Edouart AS, Hondermarck H, Nurcombe V & Le Bourhis X. FGF signals for cell proliferation and migration through different pathways. *Cytokine & Growth Factor Res.* **11**, 295-302 (2000).

[18] Fibbi G et al. Functions of the fibrinolytic system in human Ito cells and its control by basic fibroblast and platelet-derived growth factor. *Hepatology* **29**, 868-878 (1999).

[19] Dickson IR. Bone. *Connective Tissue and Its Heritable Disorders: Molecular, Genetic, and Medical Aspects*. Royce PM & Steinmann B (eds). (Wiley-Liss, Inc., New York, 1993).

[20] Anand-Apte B & Zetter B. Signaling mechanisms in growth factor-stimulated cell motility. *Stem Cells* **15**, 259-267 (1997).

[21] Bachmann F. The plasminogen-plasmin enzyme system. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice, Third Edition*. Colman RW, Hirsh J, Marder VJ & Salzman EW (eds). (J.B. Lippincott Company, Philadelphia, 1994).

[22] Sherry S & Marder VJ. Therapy with antifibrinolytic agents. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice, Third Edition*. Colman RW, Hirsh J, Marder VJ & Salzman EW (eds). (J.B. Lippincott Company, Philadelphia, 1994).

[23] Ellis et al. *Plasminogen activation in fibrinolysis, in tissue remodeling, and in development* **667** Brakman P & Kluft C (eds). (Annals of the New York Academy of Science, New York, 1992).

[24] Salvesen G & Pizzo SV. Proteinase inhibitors: α-macroglobulins, serpins, and kunins. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice, Third Edition.* Colman RW, Hirsh J, Marder VJ & Salzman EW (eds). (J.B. Lippincott Company, Philadelphia, 1994).

[25] Gebhard W, Tschesche H & Fritz H. Biochemistry of aprotinin and aprotinin-like inhibitors. *Research monographs in cell and tissue physiology **12** Proteinase inhibitors.* Barrett AJ & Salvesen G (eds). (Elsevier, New York, 1986).

[26] Mintz PD et al. Review: fibrin sealant: clinical use and the development of the University of Virginia Tissue Adhesive Center. *Annal. Clin. Lab. Sci.* **31**, 108-117 (2001).

[27] Ikegami S et al. Aphidicolin prevents mitotic cell division by interfering with the activity of DNA polymerase-a. *Nature* **274**, 458-460 (1978).

[28] Dellenback RJ & Chien S. The extinction coefficient of fibrinogen from man, dog, elephant, sheep, and goat at 280 mμ. *Proc. Soc. Exp. Biol. Med.* **134**, 353-355 (1970).

[29] Murphy G, Atkinson S, Ward R, Gavrilovic J, Reynolds JJ. The role of plasminogen activators in the regulation of connective-tissue metalloproteinases. *Annal. New York Acad. Sci.* **667**, 1-12 (1992).

[30] Hoffmann JJML & Vijgen M. Prevention of in vitro fibrinogenolysis during laboratory monitoring of thrombolytic therapy with streptokinase of APSAC. *Blood Coagul. Fibrinolysis* **2**, 279-284 (1991).

[31] Pierschbacher MD & Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* **309**, 30-33 (1984).

[32] Hajjar KA & Nachman RL. The human endothelial cell plasmin-generating system. *Hemostasis and Thrombosis: Basic Principles and Clinical Practice, Third Edition.* Colman RW, Hirsh J, Marder VJ & Salzman EW (eds). (J.B. Lippincott Company, Philadelphia, 1994).

[33] Pohl J & Christophers E. Growth characteristics of skin fibroblasts and 3T3 cells entrapped by polymerizing fibrin. *In Vitro* **15**, 624-630 (1979).

[34] Tomasek JJ, Hay ED & Fujiwara K. Collagen modulates cell shape and cytoskeleton of embryonic corneal and fibroma fibroblasts: distribution of actin, α-actinin, and myosin. *Develop. Biol.* **92**, 107-122 (1982).

[35] Pelham RJ, Jr. & Wang Y. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc. Natl. Acad. Sci. USA* **94**, 13661-13665 (1997).

[36] Lo C, Wang H, Dembo M & Wang Y. Cell movement is guided by the rigidity of the substrate. *Biophysical J.* **79**, 144-152 (2000).

[37] M. Lütolf, A. Metters & J. Hubbell, unpublished results.

[38] DiMilla PA, Stone JA, Quinn JA, Albelda SM & Lauffenburger DA. Maximal migration of human smooth cells on fibronectin and type IV collagen occurs at an intermediate attachment strength. *J. Cell Biol.* **122**, 729-737 (1993).

[39] Palecek SP, Loftus JC, Ginsberg MH, Lauffenburger DA & Horwitz AF. Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature* **385**, 537-540 (1997).

[40] Schense JC & Hubbell JA. Three-dimensional migration of neurites is mediated by adhesion site density and affinity. *J. Biol. Chem.* **275**, 6813-6818 (2000).

CHAPTER 5

IN VIVO BONE FORMATION

Recombinant human bone morphogenetic protein-2 (rhBMP-2) was added to hydrogel precursors and noncovalently entrapped within hydrogels during gel cross-linking by conjugate addition. Hydrogel disks were implanted in critical size calvarial defects in rats. After 1, 3, and 5 wk implantation times, animals were sacrificed, and the defect regions explanted, radiographed, and histologically processed. Plasmin-degradable materials were infiltrated by cells, predominantly fibroblast-like cells, in a time dependent manner. At a dose of 5 μg BMP-2 per implant, intramembranous bone formation was observed, and the amount of mineralized bone increased as a function of implant duration. By 5 wk, implant materials were mostly resorbed, and new bone covered on average 80% of the defect area. Control materials, either containing no growth factor or made with a plasmin-insensitive cross-linker, showed no cell infiltration and no bone formation beyond the wound edges. rhBMP-2 was also incorporated into plasmin-sensitive hydrogels containing a heparin-based growth factor delivery system. After 3 wk, new bone covered 94% of the defect area and radio-opacity was 84% of that of neighboring, uninjured bone.

INTRODUCTION

Current clinical setting

Insufficient bone formation may result from congenital malformations, nutritional or metabolic abnormalities, or diseases such as periodontitis and rheumatoid arthritis. Following injury, bone naturally has a high capacity to regenerate, and 90-95% of fractures heal spontaneously. However, healing can be impaired by insufficient immobilization of the fracture, distraction of fracture fragments, repeated or excessive manipulation of the fracture, poor bone stock, damage to surrounding soft tissue, infection, and tissue necrosis. Impaired healing can result in a delayed union or a nonunion. A nonunion is an absence of continuous and functional bone. Loss of large segments of bone due to trauma or resection of large volumes of bone due to infection or malignancy would benefit from enhanced rates of healing or assistance in healing in order to help patients regain limb functionality and mobility.[1] Economically, the estimated annual market in the United States exceeds $500 million for more than 200,000 bone grafts,[2] predominantly for delayed unions and nonunions, and more than one million surgical procedures for bone indications.[3] When one considers dental indications, repairs of congenital defects, osteoporosis, and veterinary indications, the market could reach a multi-billion-dollar level.[2]

The autograft, typically from the anterior iliac crest (the rear of the pelvic bone), is the standard treatment for bone defects and nonunions. It provides a substrate, growth factors, and sometimes also a good source of osteocompetent cells or osteoblasts; the osteoconductive activity of the graft depends on how the bone is handled during the transplantation process. Nevertheless, grafting is not always successful; for example, as many as 45% of spinal fusion grafts fail. Also, autografting is limited by supply, donor site

pain and morbidity (10-20% of autografts[4]), donor site infection, and blood loss during harvest. To overcome these drawbacks of bone autografts, allografts from treated human cadaver bone have been used. Allografts provide a scaffold and growth factors, but allografts are less osteoinductive than autografts due to their acellularity and reduced content of osteoinductive factors following demineralization and lipid extraction. Also, allografts and xenografts carry potential risks of disease transmission (e.g., HIV, hepatitis, and BSE/Creutzfeld-Jacob disease) as well as immune activation and rejection if the bones are not extracted properly.

Already there is a transformation underway in orthopedic surgery away from bone grafts and substitutes and toward tissue engineered bone where exogenous bone promoting factors play an active role.[2] Preclinical and clinical trials involving rhBMP-2 (Genetics Institute),[5] BMP-7 (Novos™, Creative Biomolecules),[6] and NeOsteo™ (Sulzer Biologics), a purified bovine bone extract of growth factors, have been underway since the early 1990s. In these cases appropriate biological factors have been identified and have been combined with the best available matrices to date. However, significant patient variation in trials with rhBMP-2 and BMP-7 has been observed and this may in large part result from current matrix limitations.[7]

Biology of bone repair

Bone repair generally proceeds via one of two pathways: endochondral or intramembranous bone formation. Endochondral bone formation is observed in normal fracture repair. It passes through an initial cartilagenous stage which is later mineralized and transformed by osteoblasts and osteoclasts to bone. By contrast, intramembranous bone formation occurs by direct differentiation of mesenchymal cells or immature fibroblasts to

osteoblasts and is usually observed during graft incorporation. The bone formation pathway is also a function of the injury or implant site, the concentration of osteoinductive factors (higher doses favoring intramembranous bone formation), and the nature of the factor delivery system.[8] Independent of pathway, regeneration of bone requires three things: bone-forming or osteoprogenitor cells; osteoinductive factors; and a scaffold or matrix that is inherently or can be rendered osteoinductive.[9]

Osteoprogenitor cells

In the absence of sufficient osteoblasts, bone producing cells, at a bony defect site, osteoprogenitor cells are required for bone formation. Osteoprogenitor cells are cells that can become bone cells. The exact origin, source, and nature of these cells are still debated. One cell type that has been implicated as a predominant osteoprogenitor is the mesenchymal (stromal) stem cell.[9, 10] Typically it is believed that progenitor cells migrate by chemotaxis from bone, bone marrow, periosteum, and vasculature to a site where bone will be formed following an initial inflammatory response at the injury site. At the site, the cells undergo mitogenesis and differentiation. They deposit osteoid which mineralizes to woven bone and should then be remodeled by osteoclasts and osteoblasts.[11]

Normally patients endogenously possess osteoprogenitor cells. However, some of the cells may be lost from the site of injury due to severe trauma. In such cases, osteoblasts or osteoprogenitors may have to be grafted or transplanted. If possible, it is most desirable to expand endogenous cells *in vitro* and then reimplant them in larger numbers. However, some patients may have fewer progenitor cells due to aging, osteoporosis, metabolic disorders, chemotherapy or irradiation. Also the progenitors' activity may vary by patient again due to age, disease, and drug treatment (e.g., with nonsteroidal anti-inflammatory drugs,

corticosteroids, or chemotherapy) and limit the rate and quality of bone formation. For such patients, other forms of cell-based therapy may be required in order to provide adequate bone quantity and quality.

A number of cell types are of interest for cell-based tissue engineering of bone: osteoblasts, chondrocytes, bone marrow cells, mesenchymal stem cells, and genetically modified cells. While osteoblasts are ultimately the desired phenotype for bone formation, they are difficult to obtain and to expand in culture with preservation of phenotype.[11] Chondrocytes have been evaluated for their role in endochondral bone formation;[12] nevertheless, their application suffers from the same drawbacks as the use of osteoblasts. Bone marrow contains osteoprogenitor cells, but even in healthy bone marrow these progenitor cells are available only in low concentrations, 0.001% of nucleated cells, so the osteoinducing potential of bone marrow is relatively low.[11]

Mesenchymal stem cells from bone marrow and periosteum can be isolated and expanded extensively without loss of phenotype and differentiation potential. They have multilineage developmental potential to form bone, bone marrow, cartilage, tendon, muscle, fat, and skin, but to differentiate they require a signal, such as an osteoinductive factor.[11] With cell-based therapies, one can delivery in the same package cells and the osteoinductive factors they need for differentiation, and this approach is being evaluated in *in vitro* and *in vivo* bone formation assays. Lieberman et al.[13] have transfected marrow-derived cells *in vitro* with the cDNA for rhBMP-2 using adenoviral delivery and used the cells to heal femoral defects in rats. Viggeswarapu et al.[14] have similarly transfected marrow-derived cells with the cDNA for LIM mineralization protein-1 (LMP-1) and observed spinal fusion following their delivery to rabbits.

Osteoinductive factors

Osteoinductive factors include bone morphogenetic proteins, growth factors, differentiation factors, and polypeptide hormones that are expressed and presented to cells in a temporally and spatially organized manner to induce bone formation. Bone morphogenetic protein, the prototypic osteoinductive factor, was first described in theory in 1965 when it was observed that demineralized bone induced ectopic bone formation in rodents.[15] Since then, fifteen BMPs have been identified.[7] BMP-1 is structurally unrelated to the other BMPs which belong to the TGF-β superfamily of related peptide growth factors.[16] The rest of the BMPs are active as homo- and heterodimers. They are chemoattractants for bone cells and osteoprogenitors, and they are morphogens (osteoinductive) rather than a mitogens (osteogenic) as they induce differentiation rather than proliferation.[2] They initiate bone formation by both endochondral and intramembranous pathways.[17] Osteoblasts and fibroblasts have BMP receptors[18] although it is not clearly established if those cell types respond to BMPs; it may be that fully differentiated cells are not stimulated by BMPs and that BMP influence is limited to immature or multipotent cells.[7] During embryogenesis, growth, and healing, BMPs induce mesenchymal stem cells to become chondroblasts and osteoblasts.[19]

Isolation of BMP from animal sources provides relatively small quantities due to low physiologic concentrations and poor recovery. The only commercial source of extracted bovine BMP is available as NeOsteo™ by Sulzer Biologics and is being investigated in human clinical trials. Recombinant human BMPs have been expressed[20] and are available in relatively large, highly purified quantities. Purified BMP-2 through BMP–7 are osteoinductive.[7] BMP-2, BMP-4, BMP-5, and BMP-7 (osteogenic protein-1, OP-1) induce

bone formation, including marrow formation, in the absence of other BMPs[8] and hence can be used as single factors to initiate the entire cascade of bone formation.[21] BMPs are the only differentiation factors that have been identified and demonstrated to single-handedly induce *de novo* bone formation both *in vivo* and *in vitro*, and of them, BMP-2 is the most osteoinductive.[16]

Many factors other than BMPs have been implicated in the bone formation process although their roles are less well understood than those of the BMPs and none single-handedly induces bone formation. TGF-β1 may enhance the activity of BMP-2 *in vivo* in a concentration dependent manner; however, it reduces the activity of matrix degrading enzymes and hence inhibits cell migration and recruitment required for bone formation. Growth hormone and insulin-like growth factor-1 are important in skeletal development and growth, but their roles in healing are not understood.[22] LMP-1 is an intracellular protein which induces membranous bone formation in transfected bone marrow cells and is part of a signaling cascade which may induce expression of BMPs and their receptors.[2] Vitamin D and parathyroid hormone (PTH) regulate bone formation and resorption by regulating calcium and phosphorus movement between bone and serum.[23] Selection of appropriate osteoinductive factors for therapy is complicated by the fact that at different sites and in different types of defects, different factors may be required for functional healing. For example, segmental long bone defects require cortical bone formation while spinal fusion may require cancellous (membranous) bone.

Delivery of osteoinductive factors

Initial osteoinductive factor delivery was a bolus injection after which most of the protein was not retained at the delivery site. For this reason, and the fact that humans require

higher doses of osteoinductive factors than lower animals, relatively high doses of BMPs (mg in humans) had to be delivered in order to obtain an effect at the treatment site.[2] However, the use of such high doses and the tendency of most of the protein to diffuse away from the treatment site gave rise to fears of inflammation and bone formation beyond the treatment site. Numerous continuous or delayed release systems for proteins and specifically for bone formation have since been investigated. Several take advantage of the fact that BMPs were initially isolated from a collagenous bone matrix and bind collagen, possibly by hydrophobic interactions. However, the mean residence time for rhBMP-2 in Helistat®, a resorbable bovine collagen hemostatic sponge (Integra LifeSciences), is less than 7 hr. However, osteoinduction is correlated with BMP retention time, and thus there is a need for carriers to delay dispersion of otherwise diffusible BMPs.[24]

Growth factors, such as members of the FGF, vascular endothelial growth factor, and TGF-β families, are sequestered to the extracellular matrix not only by binding collagen, but also by binding heparin and heparan sulfate. BMPs, as members of the TGFβ family, also have heparin-binding domains which are located at their amino-termini where the first seventeen amino acid residues contain 10 basic residues (Lys, Arg, and His).[25] This heparin-binding character of the proteins can be used to sequester them within synthetic biomaterials.[26] For example, heparin binding peptides can be covalently bound to fibrin during its cross-linking by factor XIIIa. A molar excess of heparin binding peptides can electrostatically immobilize heparin within the matrix, and the heparin can simultaneously bind heparin-binding growth factors. The result is a sponge of growth factor where passive diffusion of growth factor out of the material is minimized, and release is instead triggered by cellular activity and enzymatic activity, e.g., by heparinase and plasmin. Such a delayed

release system maintains a high local concentration of growth factor for controlled delivery to the implant site and should minimize systemic side effects. Also, its design principles can be applied to other biomaterials.

The use of gene therapy to delivery cDNA sequences of osteoinductive proteins rather than delivering the protein itself are also being investigated and may potentially be cheaper than protein production and purification for large doses of protein. Theoretically, gene therapy for bone formation is less challenging than for diseases such as cystic fibrosis and muscular dystrophy that are also targets of gene therapy; in those cases, high transfection rates and life-long expression are desired. For bone formation, diffusion of osteoinductive factors means transfection rates need not be maximized. *In vitro* transfection and delivery within a matrix minimize the problem of targeting. And finally, expression can be relatively transient, so transfection need not be stable.[2] The challenges are to find the an optimal vector and dose that do not cause inflammatory and adverse immune reactions.

Osteoinductive matrices

Whether one attempts to treat bone defects with cellular techniques or with molecular, e.g., protein delivery, techniques, or both, an osteoinductive matrix is required. An osteoinductive matrix or scaffold must support infiltration by osteoinductive cells as well as neovascularization by providing a cell adhesive substrate. It should be temporary so that it is replaced by native bone and does not cause long term inflammation or other immune response. If possible, it should favor differentiation of cells to an osteoblastic phenotype.

Some naturally derived materials have been used clinically. Demineralized bone still contains active osteoinductive factors and has been modified to carry exogenous factors; however, its supply is limited and carries risks like other transplants. Collagen formulations

have had some success due to the ability of collagen to retain some BMP, after an initial burst release, and are undergoing preclinical trials (rhBMP-2 in Helistat).[5] Collagen sponges are pliable and can be cut and molded to fit various defect geometries; however, depending on the defect, the sponges may not remain fixed in place.

Because the hydroxyapatite form of calcium phosphate is naturally found in bone matrix, ceramics of hydroxyapatite as well as β-tricalcium phosphate and calcium sulfate have been widely evaluated as osteoinductive biomaterials. Hydroxyapatite is resorbed very slowly; β-tricalcium phosphate and calcium sulphate are resorbed more rapidly but have shown some inflammatory response. These classes of ceramics are osteoconductive; they support cell adhesion, differentiation, mineralization, and angiogenesis. However, these activities are isolated to their surfaces only. A partial solution to this problem has been to make porous ceramics, and optimal pore sizes in hydroxyapatite are 300-400 μm.[7] When synthesizing porous ceramics, it has been difficult to create non-brittle materials with interconnected pores for continuous bone formation. Another drawback of ceramics is their radio-opacity which makes evaluation of the repair process difficult.[11] Other classes of inorganic materials, bioactive glasses and glass-ceramics of SiO_2, Na_2O, CaO, and P_2O_5 in certain ratios are also being evaluated for bone repair, especially since they bind tightly to tissue by formation of a hydroxy carbonate apatite layer.[7]

The use of absorbable polymeric materials for orthopedic applications has been reviewed by An et al. Briefly, poly(α-hyroxy esters) such as poly(lactic acid), poly(glycolic acid), and their copolymers, PLGA foams and fiber meshes, are being broadly evaluated. However, they undergo bulk degradation with premature loss of mechanical properties, must be surface treated for cell attachment, and elicit an inflammatory tissue response, presumably

due to crystalline degradation products.[27] A class of tyrosine-derived polycarbonates are also based on natural metabolites but do not degrade into crystalline particles like the poly(α-hyroxy esters) because they are amorphous and do not degrade into acidic products like the poly(α-hyroxy esters).[28] Polypropylene fumarates,[29] functionalized polyphosphazenes with backbones of alternating nitrogen and phosphorous, and polyanhydrides are also being evaluated for potential osteoconductivity and biocompatibility. Compared to naturally derived biomaterials, these synthetic materials reduce the risks of immunogenicity and disease transmission, and by being degradable they should avoid the production of wear particles that result from nondegradable polymeric implants. Their supplies are generally not limited and syntheses can be tailored to meet clinical needs once established.

Models of bone formation

In vitro

In vitro, mineralized nodules can be formed by cells of the osteoblastic lineage. When examined radiographically and histologically, the nodules may exhibit gross morphological and structural characteristics of bone formed *in vivo*, specifically embryonic or woven bone.[30] With appropriate assays, such as immunohistochemistry and reverse trasncription-polymerase chain reaction (RT-PCR, Taqman), biochemical markers of bone may also be observed *in vitro* and include alkaline phosphatase activity, bone sialoprotein, type I collagen, osteopontin, and osteocalcin.[31] Finally, mineralization in the form of calcium and phosphorus deposition and apatite formation can be quantified. However, *in vitro* bone formation techniques are not yet at the stage where they can be exploited for making implants; they are currently most useful for evaluating cytocompatibility of potential implant

materials and for identifying factors that regulate proliferation and differentiation of osteoblastic precursor cells.[30]

In vivo

Two general classes of bone formation assays exist *in vivo* – heterotopic bone formation and orthotopic bone formation. In heterotopic bone formation assays, implants are placed in muscle or in other ectopic, non-bony sites. The heterotopic assays can be used to evaluate relative osteoinductive activity of factors and relative osteoconductivity of materials, and bone formation can take place by either endochondral or intramembranous pathways.

Orthotopic bone formation assays are performed in bony defect sites and are considered more clinically relevant.[16] To clinically treat bone loss from large amounts of skeleton and nonunions, one must use a model that does not exhibit spontaneous healing and preferably one which does not heal or heal well with a lesser treatment than the one being evaluated. Such a defect is called a critical size defect.

Critical size defects

A critical size defect is defined as the smallest wound that does not heal by spontaneous bone formation in the lifetime of the animal although it would if the wound were smaller.[1] The critical size defect is distinct from a nonunion where the gap may be small enough for healing but some pathologic process inhibits healing.

Craniomandibulofacial defects

Craniomandibulofacial defects are widely accepted models of intraosseus bone formation, and a hierarchy of animal models is well defined.[32] It must be recognized that healing rates slow with progress along the phylogenetic scale towards humans. Age also slows healing rates, so adult animals should be used.[1]

Intraosseus bone formation models begin with an 8 mm diameter calvarial defect in the rat.[32] This is a critical size defect in a relatively inexpensive animal and does not require large quantities of material. Also, it is tolerant to gel and particulate materials.[1] The calvaria is relatively easily accessed and does not require fixation. Defects are reproducible, and the model is well studied such that healing with and without treatments is well described in the literature. For example, addition of 6.5 mg BMP-2 to an insoluble collagenous bone matrix was shown to restore bone to a critical sized calvarial defect (80% radio-opacity after 21 days) but often with bony projections beyond the plane of the brain contour.[33] At 10 μg or less BMP-2 per implant, insoluble collagenous bone matrix induced a better response than PLGA-based delivery systems which had a mild inflammatory response,[27] although higher doses negated statistical differences.

In the hierarchy of animal models for intraosseus bone formation, the 8 mm diameter calvarial defect in the rat is followed by a 15 mm diameter calvarial defect in rabbits, which are again relatively inexpensive. The rabbit model is used for confirmation of efficacy in a second species before progressing to mandible defects in dogs which is requires more material in a "functional" or force-bearing bone, and the animals are more expensive.

Long bone segmental defects

Long bone segmental defect models are initiation with a 5 mm critical size defect in rat femurs, followed by 2 cm critical size defects in rabbit ulnae which conveniently do not require external fixation. The next model, a 2.5 cm femoral defect in sheep, does require fixation. In nonhuman primates, a 2 cm ulnar defect is the standard model for long bone segmental defects. In general the long bone models are less well characterized than the craniomandibulofacial models but they perhaps more closely mimic segmental bone loss in humans.[1]

Nonunions

Few models of nonunions exist in small animals. Nonunions may be induced by mechanical manipulations and require long manipulation times which is unpleasant for the animals and laborious for experimenters. Nonunions caused by other healing impairments are being evaluated. The include metabolic manipulation or other biochemical manipulations such as removal of proprioceptors from bone prior to injury.[1]

MATERIALS AND METHODS

Precursor synthesis

PEG-divinylsulfone (PEG-diVS) was synthesized according to the methods in Chapter 2. The peptides in Table 1 were synthesized according to the methods in Chapter 3. rhBMP-2 was produced in *E. coli* and kindly provided by Franz Weber (Klinik für Kiefer- und Gesichtschirurgie, Universitätspital Zürich).

Table 5. 1 Peptides used to form hydrogels for calvarial implantation

Sequence	Abbreviation
AcGCGYGRGDSPG-NH_2	RGD
GCYKNRCYKNRCG-NH_2	C3
GCY-*D*LysN-*D*ArgCY-*D*LysN-*D*ArgCG-NH_2	*D*C3
AcGCGK-βA-FAKLAARLYRKA-NH_2	CATIII[34]

Hydrogel preparation without a heparin-based growth factor delivery system

Hydrogels were prepared the night prior to a morning surgery. HEPES buffered saline (HEPES BS: 50 mM N-[2-hydroxyethyl]piperazine-N'-[4-butanesulfonic acid] (13.02 g/L), 137 mM NaCl (8 g/L), pH 7.6 ± 0.2) (95 μL) was added to 15. 8 mg PEG-diVS. After dissolution, the PEG-diVS solution was added to 1.2 mg RGD and allowed to sit for 1-2 min. The PEG-diVS and RGD solution was then added to 15 μL rhBMP-2 (2 mg/mL, pH 2 in 1 mM HCl). This solution was then added to 4.3 mg C3 or *D*C3. In control materials containing no rhBMP-2, 15 μl HEPES BS was added in the place of the growth factor. Aliquots (20 μL) of the precursor solutions were pipetted onto sterile, silylated (SigmaCote, Sigma, St. Louis, MO, USA) glass slides. Rectangular 700 μm-thick teflon sheets were placed at each end of the slides without touching the samples. A second sterile silylated glass slide was clamped on top of the lower slide containing the samples and teflon spacers using ordinary binder clamps (Figure 4. 1). Hydrogels were cured for 60-90 min at 37° C, 5% CO_2, and ≥ 95% relative humidity in a standard cell culture incubator. Hydrogels were then transferred with a sterile spatula to 12-well plates where each gel was swollen in 500 μL phosphate buffered saline (PBS: 2.7 mM KCl (0.2 g/L), 1.5 mM KH_2PO_4 (0.2 g/L), 137 mM NaCl (8.0 g/L), 8 mM $Na_2HPO_4 \cdot 7H_2O$ (2.16 g/L), pH 7.4) overnight.

Hydrogel preparation with a heparin-based growth factor delivery system

Hydrogels were prepared the night prior to a morning surgery. rhBMP-2 (12 μL, 2 mg/mL in histidine buffer) was mixed with heparin solution (13.2 μL, 2.5 mg/mL, 18 kDa heparin, Sigma H-9399) in a 1:1 molar ratio of heparin to rhBMP-2 monomer. The resulting solution was added to CATIII (0.35 mg, aliquoted and lyophilized previously) to give a 100:1 molar ratio of CATIII to heparin. HEPES BS (84.8 μL) was added to 15.8 mg PEG-diVS. PEG-diVS solution was added to 1.2 mg RGD and mixed. The solution containing PEG-diVS and RGD was added to the solution containing rhBMP-2 and mixed. The resulting solution was added to 4.3 mg C3 and mixed. Aliquots (50 μl) were made on sterile silylated glass slides and cured as above. Each gel was swollen in 1 mL PBS overnight. Before implantation, a sterile 8 mm biopsy punch was used to reduce the size of the gels to the size of the defect.

Rat cranial surgery

Adult female Sprague-Dawley albino rats (300–350 g) were cared for according to the Veterinary Authority of the canton of Zürich and Swiss Federal Law (Nr. 152/1997). They were housed in groups of four animals or fewer, fed with a standard diet, and acclimated for one wk prior to surgery. Animals were placed and maintained under general anesthesia (Halothane). The implant site was shaved and prepared with Betadine. A linear incision was made from nasal bone to the midsagital crest. Soft tissues were reflected, and the periosteum was dissected from the site, specifically from the occipital, frontal, and parietal bones. An 8 mm diameter craniotomy defect was created in the parietal bone with a trephine in a dental handpiece. The calvarial disk was dissected away while avoiding dural

perforations. The surgical site was flushed with saline to remove bone debris. Then a pre-formed sample disk of 8 mm was placed into the defect using a sterile metal spatula or forceps. The wound was closed by closing of the soft tissues with skin staples. Before waking, animals were given analgesia (subcutaneous injection of Buprenorphine, 0.1 mg/kg).

Animals were asphyxiated 7, 21, or 35 days after implantation. Craniotomy sites and approximately 5 mm contiguous bone were explanted and stored in 40% ethanol. At all steps, the surgeon was blinded with regard to defect treatment. The number of animals per treatment was 3-5.

Radiography

Explants were radiographed using a dental radiography unit and dental films. The radiographs were photographed and digitally scanned. The digital images were processed for area measurements of new bone formation and unfilled defect area and for quantitative grey scale analysis using a Leica QWin image analysis software package. Grey scale values of defects and normal bone were normalized to white and black values within each radiograph and were used as indicators of radio-opacity.

Histology

Samples were sequentially dried: 40% ethanol (2 d), 70% ethanol (3 d), 96% ethanol (3 d), and 100% ethanol (3 d). Dried samples were defatted in xylene (3 d). Defatted samples were saturated (3 d) with methylmethacrylate (MMA, Fluka 64200) and then fixed at 4 °C by soaking (3 d) in MMA containing di-benzoylperoxide (20 mg/mL, Fluka 38581).

Fixed samples were embedded in MMA, di-benzoylperoxide (30 mg/mL), and 100 μL/mL plastoid N or dibutylthalate (Merck) at 37 °C. Sections (5 μm) were stained with Toluidine blue O and Goldner Trichrome. Histologic slides were scanned and the digital images processed with Leica QWin software.

Statistics

Values are given as means ± average deviations. P values were calculated using a two-tailed, non-paired Student t test.

RESULTS

Gross and radiographic evaluation

In injured animals receiving no implant, no bone formation was observed at 3 wk. In animals receiving hydrogels that did not contain rhBMP-2, bone formation occurred at the wound edges but did not significantly penetrate the implant. In animals receiving implants containing 5 μg rhBMP-2 and cross-linked with *D*C3, a plasmin-insensitive peptide, bone formation was again observed only at the wound edges. In animals receiving implants containing 5 μg rhBMP-2 and C3, the plasmin-degradable cross-linker, significant bone formation was observed at 3 wk compared to controls ($p < 0.01$) (Table 5. 2; Figure 5. 1). For animals receiving C3 implants containing CATIII and heparin, bone formation was significantly greater than controls and implants without CATIII and heparin ($p < 0.02$).

Table 5. 2 Effect of treatment on bone formation at 3 wk

Material	Growth factor	Radiograph area of new bone/area of defect	Radio-opacity of new bone/radio-opacity of uninjured bone
C3	none	0.18 ± 0.04	0.41 ± 0.08
*D*C3	5 μg rhBMP-2	0.11 ± 0.03 *	*
C3	1 μg rhBMP-2	0.15 ± 0.04 *	*
C3	5 μg rhBMP-2	0.73 ± 0.07	0.47 ± 0.10
C3 + CATII/heparin	5 μg rhBMP-2	0.94 ± 0.04	0.84 ± 0.13

* radiographs not taken; area estimates made by the surgeon at time of explant

Bone formation in C3, plasmin-degradable, hydrogels (5 μg rhBMP-2/implant) was observed as a function of the duration of implantation. Radio-opacity and the area of implant filled with new bone increased with time although the increase from 3-5 weeks was not significant ($p = 0.16$ and 0.33, respectively) (Table 5. 2; Figures 5. 2 and 5. 3).

Table 5. 3 Bone formation in plasmin-degradable hydrogels as a function of time

Duration of implant	Radiograph area of new bone/area of defect	Normalized grey scale of new bone/grey scale of uninjured bone
1 wk	0.26 ± 0.08	0.34 ± 0.08
3 wk	0.73 ± 0.07	0.47± 0.10
5 wk	0.81 ± 0.10	0.62 ± 0.12

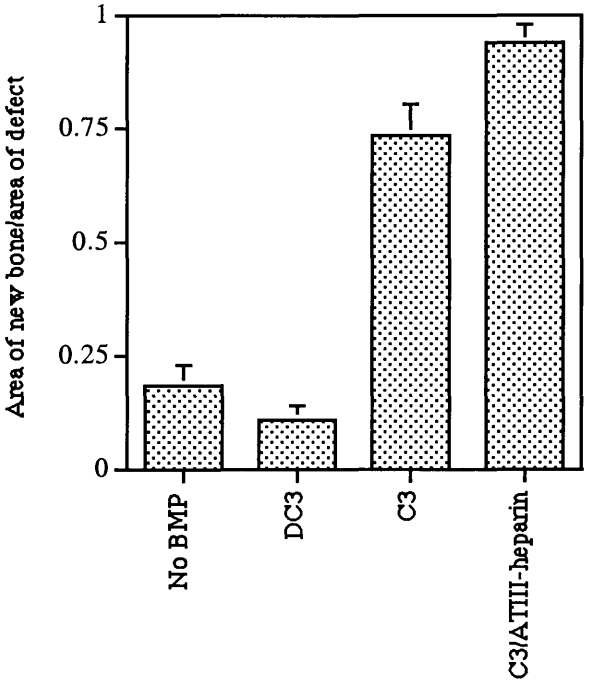


Figure 5. 1 Area of new bone at 3 wk as a function of treatment

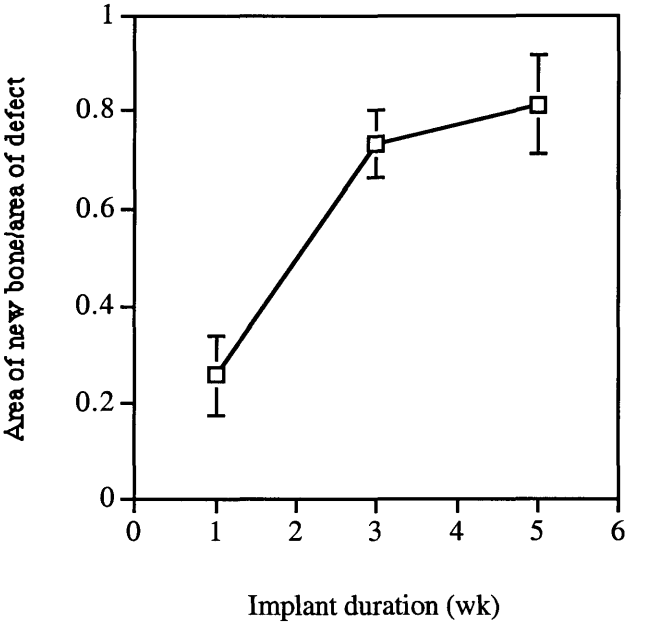


Figure 5. 2 Area of new bone as a function of implant duration

C3 plasmin-degradable materials without a heparin-based growth factor delivery system; 5 μg rhBMP-2 per implant.

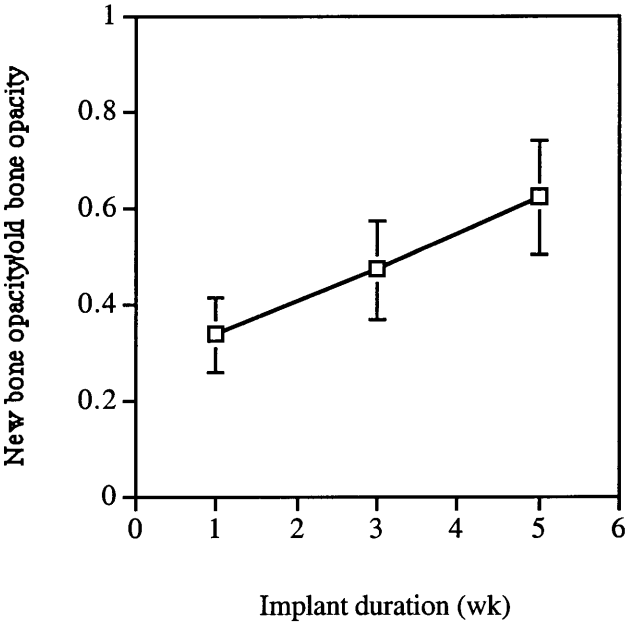


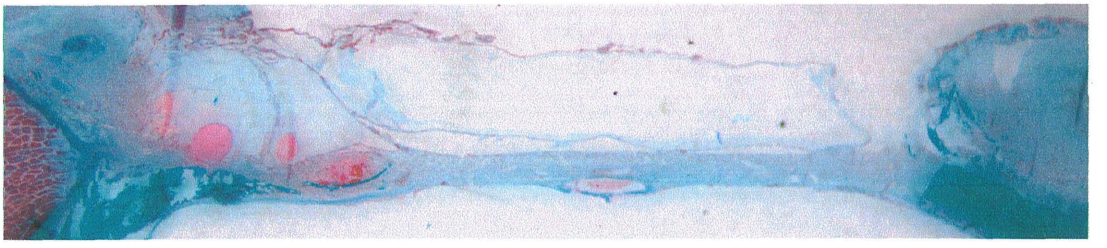
Figure 5. 3 Opacity of new bone as a function of time

C3 plasmin-degradable materials without a heparin-based growth factor delivery system; 5 μg rhBMP-2 per implant.

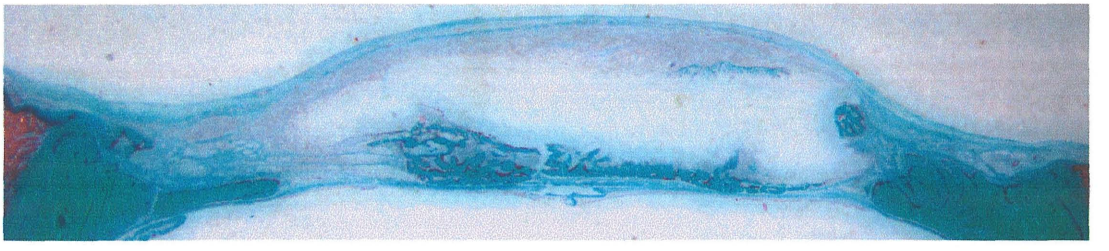
Histological evaluation

Histological preparations indicated that bone formation occurred in the following manner: fibroblast-like cells encapsulated the hydrogel implants; fibroblast-like cells from the capsule infiltrated the plasmin-sensitive and BMP-containing hydrogels but not the plasmin-insensitive hydrogels nor those lacking BMP; cells infiltrating the hydrogels were transformed into bone-producing cells starting from the perimeter of the implant and progressing inwards as a function of time (Figure 5. 4). Calcified bone could be observed with Goldner Trichrome stain (dark green), and osteoblasts (fuschia) could be observed at the osteoid interface between the newly deposited calcified bone and the fibroblast-like cells infiltrating the matrix.

a



b



c

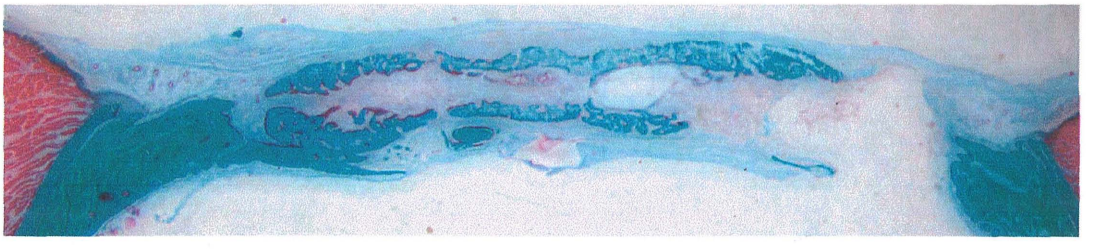


Figure 5. 4 Histological view of bone formation as a function of time

Bone formation in synthetic plasmin-degradable hydrogels containing 5 μg rhBMP-2 at gel formation. Defects were 8 mm critical size defects in rat calvariae. **a** 1 wk, **b** 3 wk, **c** 5 wk.

DISCUSSION

Synthetic hydrogels were used to induce *de novo* bone formation *in vivo*. The hydrogels were formed by conjugate addition reactions in contact with a protein, rhBMP-2, which maintained its activity and was able to induce bone formation in a critical size defect in calvariae in rats. In the absence of a specialized growth factor delivery system, e.g., simply by physical entrapment of the BMP within the gel, approximately 73% of the defect

area was covered with bone at 3 wk and 81% at 5 wk. The healing rate and extent of healing could be increased by including a growth factor delivery system such that 94% of the defect area was covered with new bone after 3 wk. In all cases, the areas remaining devoid of new bone were near the center of the defect area, implying that healing was occurring from the wound margins inward and simply required more time for 100% coverage. Implants have been made for a 12 wk time point, but the results are not yet available to prove or disprove the hypothesis.

The hydrogels used in this work are effectively nonporous: their pore sizes are smaller than the length scale of cell processes (Chapter 4). For cells to penetrate the materials, they must degrade them using the fibrinolytic cascade involving plasmin. This was observed *in vivo* where cells could penetrate hydrogel matrices formed with a plasmin-sensitive cross-linking peptide but not hydrogel matrices formed with a plasmin-insensitive cross-linking peptide containing *D* stereoisomers of lysine and arginine. These results are novel in light of the fact that other synthetic (e.g., non-biologically derived) materials that support tissue ingrowth for wound repair in general and bone formation specifically are porous, with pore sizes typically on the order of 250-500 μm. The materials used herein more effectively mimic natural extracellular matrices, which do not contain pores larger than the length scale of a cell process. The materials described herein are degraded on the time scale of cell migration which is useful because degradation that is too slow hinders bone formation, and degradation that is too rapid releases osteoinductive factors and decreases matrix mechanical properties prematurely, both leading to insufficient bone formation.[7]

The development of effective osteoinductive matrices that slowly and locally deliver osteoinductive factors should obviate the need for somatic gene therapy in cases where

healthy progenitor cells are present in the wound bed of a patient. Since the materials described herein also possess the capacity to be formed in contact with cells, in cases where insufficient numbers of osteoprogenitors are available in the wound bed, autologous cells could be transplanted within the materials in conjunction with an osteoinductive factor. Thus, many of the safety issues related to gene transfection and the use of transfected cells may be avoided in bone formation simply by using appropriate osteoinductive factors in conjunction with appropriate matrices.

Use of effective matrices for localized delivery of osteoinductive factors may also resolve other practical, e.g., synthetic, and safety concerns regarding the use of large doses of BMP and the diffusion of BMP away from the intended site of bone formation. If BMP can be localized within an implant until cell-mediated release due to material degradation, then systemic effects should be minimized because less BMP will diffuse out of materials; it will instead be released in a relatively slow manner and at lower concentrations such that the amounts released at a given time are all consumed locally and only exert their effects on local cells. Delivery of osteoinductive factors in a delayed, localized way should also require less of the factors in total since the effects are concentration dependent. This is particularly important in human applications since μg-mg doses of BMPs have been required with previous treatments although femtomolar concentrations induce chemotaxis and nanograms induce osteogenic effects *in vitro*.[7]

A heparin-based growth factor delivery system was adapted such that it could be incorporated into synthetic materials formed by conjugate addition reactions. To do so, a heparin-binding peptide[34] was modified similarly to its modification for incorporation into fibrin gels.[26] Instead of synthetically adding an amino-terminal sequence substrate for factor

XIIIa, a cysteine residue was added for covalent incorporation by Michael-type addition with PEG-vinylsulfones in the material precursor solution.

It remains to be proven whether the presence of the heparin and heparin-binding CATIII peptide mediated higher rates of bone formation through the proposed mechanism or whether the effect was primarily mediated by precipitation. In the presence of heparin and the heparin-binding peptide, precipitates of BMP-2, which is known to have poor solubility in biological buffers at neutral pH, could be observed during material preparation and in the resulting hydrogels. Also, PEG, the primary component of the hydrogels, is known to precipitate proteins, although such precipitation was not observed in the absence of heparin and CATIII. It is not known how the precipitated BMP interacts with the intended delivery system due to phase separation. In any case, it is hypothesized that the increased rate and extent of bone formation were due to better retention of the BMP within the hydrogel since better BMP retention is known to elicit these effects. Whether the better retention is due to the precipitation and slow dissolution of the BMP, the heparin bridge between the BMP and the hydrogel, or a combination of the two should be determined. Also, the effect of precipitation on BMP remains to be explored in full.

The effects of BMP solubility are also being considered by other researchers, and it has been suggested that lower BMP solubility improves retention in a variety of matrices.[24] It was hypothesized that the lower osteoinductive activity of BMP-4 compared to BMP-2 may be due to its lower pI and higher solubility than BMP-2. Chemical modification of BMP-2 to change its pI affected its solubility and as a result its retention. Specifically, reducing the pI of BMP-2 by succinylating it increased its solubility and decreased its retention.

The hydrogels implanted in this work were made ahead of time and implanted as pre-formed disks into a defect of simple geometry. Because the materials are formed using selective conjugate addition reactions with minimal side reactions with amines and other functional groups on cells and tissues (Chapter 2), they could also be formed *in situ* to match defect architectures that are more complicated. The materials have the advantage that they can be sterilized by sterile filtration of the precursor solutions. This is useful since ethylene oxide sterilization has a significant negative impact on BMP activity. BMP can tolerate gamma irradiation up to 5 Mrads, but matrix materials such as collagen do not tolerate the dose of 2.5 Mrads requested by the United States Food and Drug Administration.

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REFERENCES

[1] Einhorn, TA. Clinically applied models of bone regeneration in tissue engineering research. *Clinical Orthopaedics and Related Research* **367S**, S59-67 (Lippincott Williams & Wilkins, Inc. 1999).

[2] Boden, SD. Bioactive factors for bone tissue engineering. *Clinical Orthopaedics and Related Research* **367S**, S84-94 (Lippincott Williams & Wilkins, Inc. 1999).

[3] Langer R & Vacanti JP. Tissue Engineering. *Science* **260**, 920-926 (1993).

[4] Boden, SD. The role of BMPs in spine fusion: the road from pre-clinical to human clinical trials. *2nd International Conference on Applications in Orthopaedic Surgery: Bone Morphogenetic Proteins* (Lake Tahoe, CA, USA, June 7-11, 2000).

[5] Hollinger JO et al. Recombinant human bone morphogenetic protein-2 and collagen for bone regeneration. *J. Biomed. Mater. Res.* **43**, 356-364 (1998).

[6] Cook SD, Wolfe MW, Salkeld SL & Rueger DC. Effect of recombinant human osteogenic protein-1 on healing of segmental defects in non-human primates. *J. Bone & Joint Surgery* **77**, 734-750 (1995).

[7] Groeneveld EHJ & Burger EH. Bone morphogenetic proteins in human bone regeneration. *Eur. J. Endocrinology* **142**, 9-21 (2000).

[8] Wang EA et al. Recombinant human bone morphogenetic protein induces bone formation. *PNAS* **87**, 2220-2224 (1990).

[9] Boden SD, Lane JM, and Finnegan, M. Breakout session 2: bone. *Clinical Orthopaedics and Related Research* **367S**, S130-132 (Lippincott Williams & Wilkins, Inc. 1999).

[10] Scutt A, Mayer H & Wingender E. New perspectives in the differentiation of bone-forming cells. *Biofactors* **4**, 1-13 (1992).

[11] Bruder, SP & Fox BS. Tissue engineering of bone: cell based strategies. *Clinical Orthopaedics and Related Research* **367S**, S68-83 (Lippincott Williams & Wilkins, Inc. 1999).

[12] Goldstein, SA. Breakout session 2: bone. *Clinical Orthopaedics and Related Research* **367S**, S133-134 (Lippincott Williams & Wilkins, Inc. 1999).

[13] Lieberman JR, Daluiski a, Stevenson S, Wu L, McAllister P, Lee YP, Kabo JM, Finerman GAM, Berk AJ & Witte ON. The effect of regional gene therapy with bone morphogenetic protein-2-producing bone marrow cells on the repair of segmental femoral defects in rats. *J. Bone. Joint. Surg. Am.* **81A**, 905-917 (1999).

[14] Viggeswarapu M, Boden SD, Liu YS, Hair GA, Louis-Ugbo J, Murakami H, Kim HS, Mayr MT, Hutton WC & Titus L. Adenoviral delivery of LIM mineralization protein-1 induces new bone formation in vitro and in vivo. *J. Bone. Joint. Surg. Am.* **83A**, 364-376 (2001).

[15] Urist, MRI. Bone: formation by autoinduction. *Science* **150**, 893-899 (1965).

[16] Kirker-Head CA. Recombinant bone morphogenetic proteins: novel substances for enhancing bone healing. *Veterinary Surgery* **24**, 408-419 (1995).

[17] Sasano Y et al. BMPs induce direct bone formation in ectopic sites independent of the endochondral ossification in vivo. *Anatom. Record* **236**, 373-380 (1993).

[18] Paralkar VM, Hammonds RG, Reddi AH. Identification and characterization of cellular binding proteins (receptors) for recombinant human bone morphogenetic protein 2B, an initiator of bone differentiation cascade. *Proc. Natl. Acad. Sci. USA* **88**, 3397-3401 (1991).

[19] Yabu M, Takaoka K, Hashimoto J et al Ultramicroscopic aspects of the conversion of fibroblasts to chondrocytes in the mouse dorsal subfascia induced by bone morphogenetic protein (BMP). *Arch. Histol. Cytol.* **54**, 95-102 (1991).

[20] Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM & Wang EA. Novel regulators of bone formation: molecular clones and activities. *Science* **242**, 1528-1534 (1988).

[21] Wozney, JM. The bone morphogenetic protein family: multifunctional cellular regulators in the embryo and adult. *Eur. J. Oral. Sci.* **106** (suppl 1), 160-166 (1998).

[22] Bostrom MPG, Saleh KJ & Einhorn TA. Osteoinductive growth factors in preclinical fracture and long bone defects models. *Bone Grafting and Bone Grafting Substitutes* **30**, 647-658 (1999).

[23] Hercz G. Regulation of bone remodeling: impact of novel therapies. *Semin. Dial.* **14**, 55-60 (2001).

[24] Uludag H, D'Augusta D, Golden J, Li J, Timony G, Riedel R & Wozney JM. Implantation of recombinant human bone morphogenetic proteins with biomaterial carriers: a correlation between protein pharmacokinetics and osteoinduction in the rat ectopic model. *J. Biomed. Mater. Res.* **50**, 227-238 (2000).

[25] Ruppert R, Hoffmann E & Sebald W. Human bone morphogenetic protein 2 contains a heparin-binding site which modifies its biological activity. *Eur. J. Biochem.* **237**, 295-302 (1996).

[26] Sakiyama-Elbert SE & Hubbell JA. Development of fibrin derivatives for controlled release of heparin-binding growth factors. *J. Control. Rel.* **65**, 389-402 (2000).

[27] Kenley R, Marden L, Turek T, Jin L, Ron E, & Hollinger JO. Osseos regeneration in the rat calvarium using novel delivery systems for recombinant human bone morphogenetic protein-2 (rhBMP-2). *J. Biomed. Mater. Res.* **28**, 1139-1147 (1994).

[28] Choueka J et al. Canine bone response to tyrosine-derived polycarbonates and poly(L-lactic acid). *J. Biomed. Mater. Res.* **31**, 35-41 (1996).

[29] Behravesh E, Yasko AW, Engel PS & Mikos AG. Synthetic biodegradable polymers for orthopaedic applications. *Clinical Orthopaedics and Related Research* **367S**, S118-125 (Lippincott Williams & Wilkins, Inc. 1999).

[30] Beresford JN, Graves SE & Smoothy CA. Formation of mineralized nodules by bone derived cells in vitro: a model of bone formation? *Amer. J. Med. Genet.* **45**, 163-178 (1993).

[31] Hahn SE et al. Development of an in vitro screening assay for compounds that increase bone formation. *J. Biomolecular Screening* **4**, 363-371 (1999).

[32] Schmitz JP & Hollinger JO. The critical size defect as an experimental model for cranioma nonunions. *Clin. Orthop.* **205**, 299-308 (1986).

[33] Marden LJ, Hollinger JO, Chaudhari A, Turek T, Schaub RG, & Ron E. Recombinant human bone morphogenetic protein-2 is superior to demineralized bone matrix in repairing craniotomy defects in rats. *J. Biomed. Mater. Res.* **28**, 1127-1138 (1994).

[34] Tyler-Cross R, Sobel M, Marques D & Harris RB. Heparin binding domain peptides of antithrombin III: analysis by isothermal titration calorimetry and circular dichroism spectroscopy. *Protein Science* **3**, 620-627 (1994).

CHAPTER 6

SUMMARY AND DISCUSSION

Natural extracellular matrices are hydrogels composed of diverse and complex molecules that are assembled and stabilized by physicochemical processes and covalent cross-links. Extracellular matrices provide mechanical support for cells and tissues and adhesive substrates for cells, they are enzymatically degradable, and they sequester growth factors. All of these properties are bi-directional means of communication between cells and their surrounding matrix which regulate cell and tissue morphology. In an attempt to mimic these dynamic features of natural extracellular matrices by using synthetic starting materials, a new class of synthetic hydrogels was designed.

It was desirable to use synthetic starting materials in order to avoid many of the problems associated with xenogenic or human-derived materials. Such materials may be limited in supply, may have large batch-to-batch variation, may be difficult to purify, and may transmit disease. The hydrogel materials described herein are prepared from buffered aqueous solutions of synthetically prepared polymers, poly(ethylene glycols) (PEGs) and small peptides.

The hydrogels can be formed in contact with proteins and cells by using selective conjugate addition reactions. The reduced thiol is a good choice of Michael-type donor. It is a strong nucleophile at physiological pHs at which one would like to form materials *in situ* and because it is uncommon in the extracellular space where most thiols are oxidized or inaccessible. The vinylsulfone, coupled to PEG, was the Michael-type acceptor used in the majority of this work. It was selected not only for its thiol selectivity but also for the relative

hydrolytic stability of the resulting covalent bond formed by its reaction with a thiol. Hydrolytic stability was desirable to test the hypothesis that synthetic materials can be designed to be responsive to cellular activity such as cell migration, and for that, passive hydrolysis had to occur much more slowly than enzymatic hydrolysis.

The PEG-divinylsulfone used was shown not to be cytotoxic at concentrations and time scales used to form materials in contact with cells and tissues. However, cytotoxicity of PEG-divinylsulfone at longer exposure times should not be ignored. Closer evaluation of precise mechanisms for the cytotoxicity of PEG-divinylsulfone is recommended. As an alternative to using vinylsulfone to form biomaterials, one could use other Michael-type acceptors with potentially fewer cytotoxic effects and perhaps even higher thiol-selectivity. Toward this end, functional groups such as acrylates, acrylamides, and quinones are being studied.[1]

Rheometric techniques were used to observe gel-formation on the order of seconds to a few minutes, which are surgically relevant time scales. In this dissertation, the mechanical properties of hydrogel materials were held as constant as possible in order to study the effects of variables and design parameters, e.g., cell adhesion and enzymatic degradation, other than mechanical properties. However, this relative neglect of mechanical properties is not meant to imply that mechanical properties are not important in the design of biomaterials and the understanding of biological function. On the contrary, mechanical properties are important regulators of cell behavior and are an active area of biological research.

To date, the majority of cell migration research has been performed using two-dimensional cell migration assays, that is to say using cells attached to surfaces, including coated surfaces and macroporous materials, rather than cells embedded within materials.

Similarly, synthetic biomaterials for tissue engineering are typically macroporous, with pore sizes on the order of 10 μm - 1 mm. Pore sizes larger than 10 μm are used to provide rapid diffusion of nutrients, waste products, and signalling molecules, provide open space for cell migration, and to provide a large two-dimensional surface area for cell attachment. However, with the most notable exceptions of endothelial and epithelial cells, most of the cells in the body exist within and migrate through three-dimensional materials rather than on surfaces. Extracellular matrices with pore sizes larger than cell processes, which are on the order of single microns, are not observed. Therefore, three-dimensional cell culture systems where pore sizes are smaller than the length scale of cell processes are needed to model physiological cell behavior, such as migration and neurite outgrowth. The hydrogels that are the subject of this dissertation have estimated mesh sizes on the order of 10 nm.[2] This estimation, based on the radius of gyration of PEG and an estimated molecular weight between cross-links, agrees with the observations that albumin, with a radius of gyration of 30 A, freely diffuses out of the gels while non-enzymatically degradable hydrogels can not be penetrated by cells or cell processes. The observations imply that the mesh size of the hydrogels is on the order of 10-100 nm.

Physiologically, cells migrate by enzymatically clearing their own paths in a highly regulated and localized manner. In this work, materials were designed to be degradable by plasmin, one of the principle enzymes responsible for pericellular proteolysis and cell migration at sites of wound healing and matrix remodeling. Plasmin-sensitive materials were made by using peptidyl plasmin substrates as cross-linkers. To evaluate the material design principles described in this work, it was necessary to use an *in vitro* three-dimensional cell migration assay. Due to shortcomings of published assays, a relatively simple assay was

developed. Briefly, small fibrin clots containing a high density of cells were embedded within the materials of interest, and three-dimensional cell migration out of the clots into the surrounding materials was observed with standard microscopic techniques.

Cell migration within synthetic plasmin-degradable materials was observed. No cell migration was observed in synthetic materials insensitive to plasmin. Cell migration in plasmin-sensitive materials could be inhibited by soluble inhibitors of plasmin. Conversely, migration could be increased by up-regulating the plasminogen activation pathway with FGF-2 or PDGF-BB; the increased migration was independent of cell proliferation. Cell adhesion and migration within the synthetic materials was RGD-dependent as demonstrated by the concentration-dependent effect of RGD on cell migration and the inhibition of cell migration when RDG instead of RGD was incorporated into the materials. These observations indicate that both components of the material design, the enzymatic degradability and the specifically mediated cell adhesion, in this case integrin-mediated by RGD ligands, are required for cell extension and migration within three-dimensional matrices.

Maximal rates of cell migration observed were on the order of 100 μm/day. This rate is approximately 10% of that of the same cell type, fibroblasts, observed in a biological matrix such as collagen and of regenerating peripheral axons following nerve crush or severance. Therefore, while the synthetic hydrogels described herein contain the minimum biomimetic character, cell adhesion and enzymatic degradability, required for cell-migration in three-dimensional matrices, they do not support migration rates of the same order of magnitude as naturally-occurring biomaterials. There may be a number of reasons for the lower migration rates in the synthetic materials. The mechanical properties of the materials,

which were held constant at an elastic modulus of approximately 10 kPa before swelling, may not be optimal; by comparison, the elastic modulus of the biological matrix fibrin is 30-300 Pa. The synthetic materials contained RGD as the only ligand for cell attachment. Therefore, they may not present the optimal ligand or combination of ligands, or they may not present the optimal concentration of ligand. Substrate mechanical properties may not be optimal. Also, the degradability of the materials, as determined by enzyme-substrate kinetics and the network structure, may not yet be optimized for maximal rates of cell migration.

In some of the synthetic hydrogels, rates of bulk degradation were so significant as to lead to premature loss of mechanical stability of the materials. This was probably due to diffusion of active plasmin away from the cell surface, where it was predominantly activated from plasminogen in the medium, and into the material where it degraded the plasmin-sensitive cross-links without regulation. In physiological matrices, bulk degradation in advance of the cell-matrix interface is not observed due to the presence of protease inhibitors in the matrices.

One should be able to reduce the rate of bulk degradation in the synthetic materials in a number of ways. (1) Addition of a plasmin-inhibitor, either in soluble or bound form, should reduce the rate of bulk degradation, and this was observed by longer culture times before the loss of mechanical properties in cultures containing soluble aprotinin and ε-aminocaproic acid. (2) A change in network structure can reduce the rate of bulk degradation. For example, one can change the network architecture by changing the functionality or molecular weight of the precursors. (3) Changing the enzyme-substrate kinetics by changing the substrate should affect the rates of both local and bulk degradation, and importantly the ratio of the two. It is still being determined whether the kinetics of

material degradation are controlled more by material architecture or by enzyme-substrate kinetics, providing an interesting area of ongoing study.

The rate of degradation in these synthetic materials, as in fibrin, may be auto-accelerated by generation of carboxy-terminal lysines within the material upon partial degradation. Plasmin(ogen) binds to these zwitterionic lysines better than to non-zwitterionic lysines in the material backbone, thus increasing plasmin activity in an autocatalytic mechanism. One way to solve this problem, distinct from changing network structure, would be to use an arginine-substrate for plasmin or combine lysine- and arginine-substrate cross-linkers to obtain an optimal relative concentration of the two with potentially fewer zwitterionic lysines.

Synthetic plasmin-degradable hydrogels were used to induce *de novo* bone formation *in vivo*. The hydrogels were formed in the presence of an osteoinductive protein, rhBMP-2, which maintained its activity and was able to induce bone formation in critical size calvarial defects in rats. By incorporating a heparin-based growth factor delivery system into the hydrogels, bone formation was increased relative to physical entrapment of the BMP within the gels. The delivery system is designed to be biomimetic, electrostatically sequestering heparin and heparin-binding growth factors within the matrix and minimizing passive diffusion of growth factor out of the matrix.

It remains to be proven whether the heparin-based system mediated higher rates of bone formation through the proposed mechanism or whether the effect was primarily mediated by macroscopic precipitation of the BMP which occurred in the presence of the components of the delivery system and not in their absence. In either case, it is hypothesized that the increased bone formation was due to better retention of the BMP within the

hydrogels having the delivery system. And, while it remains to be better characterized, the ability to incorporate a heparin-based delivery system within synthetic cell-responsive biomaterials is of extreme interest for therapeutic drug delivery, wound healing, and tissue engineering in order to reduce required quantities of growth factors and to localize them to treatment sites. And in the future, synthetic materials that specifically sequester growth factors with peptidyl heparin-mimics instead of heparin should also be possible.[3]

The synthetic materials described in this dissertation can be used as biological tools or model systems for studying cell behavior. For example, they could be used to answer questions of which proteases are involved in migration of different cell types. Also, due to the otherwise nonadhesive character of the gels due to the presence of PEG, one could replace the Cys-RGD peptides used in this work with other thiol-containing molecules and systematically study which ligands are necessary and sufficient for mediating various cell behaviors, such as neurite outgrowth. In spite of the minimal biological information presented in these synthetic materials, e.g., plasmin-degradable cross-links and dangling RGD peptides, three-dimensional neurite outgrowth from embryonic chick dorsal root ganglia has been observed.[4] However, consistent outgrowth has not been observed, and the factors responsible for the variability have yet to be identified.

Synthetic materials formed by conjugate addition reactions are also expected to help meet otherwise unmet clinical needs. Formulations of thiol- and acrylate-containing precursors for vertebral disc repair are already in preclinical evaluation. Other potential applications have been considered: injectable matrices for healing of bony nonunions; cell-ingrowth matrices and drug depots in bone cysts; cartilage repair; treatment of burns and chronic skin ulcers; scarless skin healing; adhesions prevention; and surgical sealants.

REFERENCES

[1] J. Hubbell et al., unpublished results.

[2] Elbert DE, Pratt AB, Luetolf MP, Halstenberg S & Hubbell JA. Protein delivery from materials formed by self-selective conjugate addition reactions. *J. Control. Release* (in review).

[3] R. Schoenmakers, H. Maynard & J. Hubbell, unpublished results.

[4] A. Pratt & J. Hubbell, unpublished results.