HYALURONAN-BASED SYNTHETIC EXTRACELLULAR MATRICES –
SYNTHESSES AND APPLICATIONS

by

Monica A. Serban

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The University of Utah

August 2007
SUPERVISORY COMMITTEE APPROVAL

of a dissertation submitted by

Monica A. Serban

This dissertation has been read by each member of the following supervisory committee and by majority vote has been found to be satisfactory.

Jerald C. Hinshaw
Kubran Balagurunathan
Yan-Ting Shiu
To the Graduate Council of the University of Utah:

I have read the dissertation of Monica A. Serban in its final form and have found that (1) its format, citations, and bibliographic style are consistent and acceptable; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the supervisory committee and is ready for submission to The Graduate School.

Date

Chair/Supervisory Committee

Approved for the Graduate Council

David S. Chapman
Dean of The Graduate School
ABSTRACT

In mammalian tissues, cells are surrounded by the extracellular matrix (ECM) - a complex network of proteins, glycosaminoglycans (GAGs) and proteoglycans (PGs). The ECM regulates numerous crucial processes such as cell proliferation, differentiation, migration, and a variety of signaling events. Synthetic extracellular matrices (sECMs) were developed in an attempt to mimic the properties of natural ECMs, specifically for tissue engineering applications.

Several sECMs are now commercially available. We compared these materials side by side in a representative array of assays, to test their biological performances and user-friendliness. Our results indicate that the ECM composition and compliance greatly influence cell behavior. Based on these data, we underline the need for a paradigm shift from classical two-dimensional (2D) culturing to the more relevant, *in vivo*-like three-dimensional (3D) techniques especially for applications directly translatable to clinical applications.

Haloacetate-modified hyaluronan (HA) polymers were synthesized and characterized in an effort to provide a greater variety of sECM components. A novel approach was employed that yielded “chemically-reversed,” electrophilic HA polymers that could be readily crosslinked with a variety of available nucleophiles. The new haloacetate-modified hyaluronan materials show dose-dependent mild cytotoxic effects and appear promising for adhesion prevention or medical device coating.
A novel thiol-modified hyaluronan polymer (HASH) was also synthesized. This biomaterial is not crosslinkable, is well tolerated by cells and shows promising results in a rat arthritis model, by slowing down the disease progression rate.

sECMs emerged to be particularly suitable for applications such as drug screening. To this end, Extracel™, a hyaluronan and gelatin-based crosslinked hydrogel was used to examine the effect of α-substituted lysophosphatidic acid (LPA) analogs. LPA and its synthesizing enzyme autotaxin (ATX) were associated in numerous studies with aberrant cellular behavior, mostly associated with cancers and tumorigenicity. The compounds tested show cell-line dependent and LPA receptor-specific effect in cell proliferation assays. Further studies that address modulation of cellular invasiveness and metastatic potential are needed for a complete profiling of these chemicals.
Dedicated to all those who believed in me.
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<table>
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<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>2D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>A.U.</td>
<td>arbitrary units</td>
</tr>
<tr>
<td>Akt</td>
<td>AKT8 virus oncogene cellular homolog</td>
</tr>
<tr>
<td>ADH</td>
<td>adipic dihydrazide</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATX</td>
<td>autotaxin</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled device</td>
</tr>
<tr>
<td>CMC</td>
<td>carboxymethylcellulose</td>
</tr>
<tr>
<td>CMHA</td>
<td>carboxymethylated hyaluronan</td>
</tr>
<tr>
<td>CMHA-S</td>
<td>thiol-modified carboxymethylated hyaluronan</td>
</tr>
<tr>
<td>CMHA-SX</td>
<td>crosslinked CMHA-S</td>
</tr>
<tr>
<td>CS A</td>
<td>chondroitin-4-sulfate</td>
</tr>
<tr>
<td>CS B</td>
<td>dermatan sulfate</td>
</tr>
<tr>
<td>CS C</td>
<td>chondroitin-6-sulfate</td>
</tr>
<tr>
<td>CS D</td>
<td>chondroitin-2,6-sulfate</td>
</tr>
<tr>
<td>CS E</td>
<td>chondroitin-4,6-sulfate</td>
</tr>
<tr>
<td>CS</td>
<td>chondroitin sulfate</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<td>--------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEGDA</td>
<td>diethylene glycol dimethacrylate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DS</td>
<td>dermatan sulfate</td>
</tr>
<tr>
<td>DTNB</td>
<td>Ellman’s reagent (5,5’-dithio-bis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTP</td>
<td>3,3’-dithiopropionyl bishydrazide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DVS</td>
<td>divinyl sulfone</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDCI</td>
<td>1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide</td>
</tr>
<tr>
<td>EHS</td>
<td>Engelbreth-Holm-Swarm</td>
</tr>
<tr>
<td>EthD-1</td>
<td>ethidium homodimer 1</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GAG</td>
<td>glycosaminoglycan</td>
</tr>
<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GlcA</td>
<td>D-glucuronic acid</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>acetylated glucosamine</td>
</tr>
<tr>
<td>GlcNH2</td>
<td>D-glucosamine</td>
</tr>
<tr>
<td>GlcNSO3</td>
<td>sulfated glucosamine</td>
</tr>
<tr>
<td>GPC</td>
<td>gel permeation chromatography</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Gtn-DTPH</td>
<td>thiol-modified gelatin</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HA</td>
<td>hyaluronan</td>
</tr>
<tr>
<td>HABA</td>
<td>bromoacetate derivatized hyaluronan</td>
</tr>
<tr>
<td>HA-BODIPY</td>
<td>dipyrrromethene boron difluoride derivatized hyaluronan</td>
</tr>
<tr>
<td>HA-DTPH</td>
<td>thiol-modified hyaluronan</td>
</tr>
<tr>
<td>HAlA</td>
<td>iodoacetate derivatized hyaluronan</td>
</tr>
<tr>
<td>HAse</td>
<td>hyaluronidase</td>
</tr>
<tr>
<td>HASH</td>
<td>thiol modified hyaluronan</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HEMA</td>
<td>poly(hydroxyethylmethacrylamide)</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HPMA</td>
<td>poly(hydroxypropyl methacrylamide)</td>
</tr>
<tr>
<td>HS</td>
<td>heparan sulfate</td>
</tr>
<tr>
<td>IdoA</td>
<td>iduronic acid</td>
</tr>
<tr>
<td>KS</td>
<td>keratan sulfate</td>
</tr>
<tr>
<td>LPA</td>
<td>lysophosphatidic acid</td>
</tr>
<tr>
<td>LPA&lt;sub&gt;1&lt;/sub&gt;</td>
<td>lysophosphatidic acid receptor 1</td>
</tr>
<tr>
<td>LPA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>lysophosphatidic acid receptor 2</td>
</tr>
<tr>
<td>LPA&lt;sub&gt;3&lt;/sub&gt;</td>
<td>lysophosphatidic acid receptor 3</td>
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<tr>
<td>LPA&lt;sub&gt;4&lt;/sub&gt;</td>
<td>lysophosphatidic acid receptor 4</td>
</tr>
<tr>
<td>LPA&lt;sub&gt;5&lt;/sub&gt;</td>
<td>lysophosphatidic acid receptor 5</td>
</tr>
<tr>
<td>LPC</td>
<td>lysophosphatidyl choline</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MTS</td>
<td>methyltetrazolium salt</td>
</tr>
<tr>
<td>MWCO</td>
<td>molecular weight cut-off</td>
</tr>
<tr>
<td>NaI</td>
<td>sodium iodide</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear regulatory factor-kappa β</td>
</tr>
<tr>
<td>OA</td>
<td>osteoarthritis</td>
</tr>
<tr>
<td>P(PF-co-EG)</td>
<td>poly(propylene fumarate-co-ethylene glycol)</td>
</tr>
<tr>
<td>PAG</td>
<td>poly(aldehyde glucuronate)</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEGDA</td>
<td>poly(ethylene glycol) diacrylate</td>
</tr>
<tr>
<td>PEO</td>
<td>poly(ethylene oxide)</td>
</tr>
<tr>
<td>PG</td>
<td>proteoglycan</td>
</tr>
<tr>
<td>PGA</td>
<td>poly(glycolic acid)</td>
</tr>
<tr>
<td>PGCL</td>
<td>poly(glycolide-co-caprolactone)</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3 kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PLA</td>
<td>poly(lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PNIPAAm</td>
<td>poly(N-isopropyl acrylamide)</td>
</tr>
<tr>
<td>PPG</td>
<td>poly(propylene glycol)</td>
</tr>
<tr>
<td>PVA</td>
<td>poly(vinyl alcohol)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>S.D.</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SAMSA fluorescein</td>
<td>5-((2-(and 3)-S-acetylmercapto)succinoyl)amino) fluorescein</td>
</tr>
<tr>
<td>SD</td>
<td>substitution degree</td>
</tr>
<tr>
<td>sECM</td>
<td>synthetic extracellular matrix</td>
</tr>
<tr>
<td>TCP</td>
<td>tissue culture plate</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

I would like to express my eternal gratitude to Dr. Prestwich for rekindling my passion for science. I greatly appreciate the given opportunity to be a part of your dynamic team, the independence, great science and possibility to learn from the best. You are an extraordinary example to follow and learn from.

To my committee members, all my appreciation for making all our meetings a true learning experience. I would like to extend my special thanks to Dr. Hinshaw for all his help.

I thank my colleagues and friends from the lab for creating an extremely friendly, fun and stimulating environment for work.

I would like to acknowledge Dr. Donald Weeks and Dr. Madhavan Soundararajan from University of Nebraska, Lincoln for all their support and for believing in me when I did not.

In the end, my gratitude goes to my husband and my family – I hope I made you proud. I could not have done it without you.
CHAPTER 1

INTRODUCTION
1.1. The extracellular matrix

Mammalian tissues are comprised of a conglomerate of interconnected cells that perform similar functions within an organism. Cells can interact with each other directly or indirectly and their activity is modulated by autocrine and paracrine regulatory mechanisms. In epithelial tissues, cells are in close contact with each other. In contrast, the majority of other tissue types have cells surrounded by a complex mesh of macromolecules and proteins referred to as the extracellular matrix (ECM) (Figure 1.1). The ECM components are secreted by the cells themselves and consist mainly of glycosaminoglycans (GAGs), which are mostly covalently linked to various proteins to yield proteoglycans (PGs), and matrix proteins such as fibronectin, laminin, collagen or elastin (1).

The mammalian GAGs encompass hyaluronan (HA), chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS) and keratan sulfate (KS) (Table 1.1) (2). All GAGs are unbranched polymers consisting of repeats of [uronic acid-amino sugar]ₙ disaccharide units. With the exception of HA that can exist as an independent macromolecule, all other GAGs are present as constituents of PGs. PGs have specific serine residues that serve as anchors for GAG chains (Figure 1.2). Although they are major components of ECM, PGs are also abundant at the cell surface where they can modulate various signaling cascades. The constituent GAGs are unbranched, but can elicit great structural diversity via processes of deacetylation, epimerization and sulfation (3). These architectural changes are all tissue specific and modulated by aging and disease (4, 5). The hydrophilic structures of the GAGs (Table 1.1), facilitate the assembly of PGs in a gel-like mesh that confers compressive resistance to the ECM.
Figure 1.1. Scanning electron micrograph of rat cornea shows interconnected fibroblasts surrounded by collagen fibers. Other ECM components have been removed by enzymatic and acid treatment (adapted from (6)).
**Table 1.1.** GAG generic structures and their corresponding PGs.

<table>
<thead>
<tr>
<th>GLYCOSAMINOGLYCAN</th>
<th>PROTEOGLYCANS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hyaluronan (HA)</strong></td>
<td>aggrecan, versican</td>
</tr>
<tr>
<td><img src="image" alt="Hyaluronan Structure" /></td>
<td></td>
</tr>
<tr>
<td>Linkage: $\beta$-1,3; $\beta$-1,4</td>
<td></td>
</tr>
</tbody>
</table>

| **Chondroitin sulfate (CS)** | aggrecan, versican, decorin, syndecan, cerebroglycan, glypican, biglycan, appican |
| ![Chondroitin Sulfate Structure](image) | |
| Linkage: $\beta$-1,3; $\beta$-1,4 |

| **Dermatan sulfate (DS)** | decorin, biglycan, fibromodulin |
| ![Dermatan Sulfate Structure](image) | |
| Linkage: $\beta$-1,3; $\beta$-1,4 |
Table 1.1. (continued)

<table>
<thead>
<tr>
<th>GLYCOSAMINOGLYCAN</th>
<th>PROTEOGLYCANS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heparin/Heparan sulfate (HS)</strong></td>
<td>syndecan, cerebroglycan, glypican, prlecain</td>
</tr>
<tr>
<td><img src="image" alt="Heparin/Heparan sulfate (HS) structure" /></td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Linkage: $\alpha$-1,4; $\alpha$-1,4</td>
<td></td>
</tr>
<tr>
<td><strong>Keratan sulfate (KS)</strong></td>
<td>fibromodulin, lumican</td>
</tr>
<tr>
<td><img src="image" alt="Keratan sulfate (KS) structure" /></td>
<td><img src="image" alt="Structure" /></td>
</tr>
<tr>
<td>Linkage: $\beta$-1,4; $\beta$-1,3</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1.2. Schematic diagram of a PG-GAG attachment site, illustrating the anchoring serine residue of the core protein and the linker tetrasaccharide chain.
Fibrous structural proteins further contribute to the tensile strength of the ECM. Collagens are the most abundant proteins in mammals and are major components of cartilage, ligaments, tendons, skin and bone (7, 8). They are secreted as procollagen by connective tissue cells as well as by other cell types. After they undergo proteolytic cleavage, procollagen molecules yield collagen, which assemble into collagen fibrils 10-300 nm in diameter that can further aggregate into collagen fibers that are several micrometers in diameter. The collagen fibers are further strengthened by covalent bonds formed between lysine residues of the constituent collagen molecules. The degree of covalent crosslinking is tissue specific and it is directly proportional to the tensile strength of the tissue (i.e., collagen in the Achilles tendon is highly crosslinked).

Elastin is secreted by the ECM cells as soluble tropoelastin (9). This precursor assembles then in the extracellular space into elastic fibers and sheets. Analogously with collagen, elastin networks are further stabilized via covalent bonds between intermolecular lysine residues. The elastic fibers are interwoven with inelastic collagen fibers to protect the tissue from tearing and to limit the extent of stretching.

Fibronectin is a large glycoprotein that has architectural function by organizing the matrix and modulating cell attachment (10). The ECM fibronectin is insoluble and present as disulfide bond-crosslinked dimers. Structurally, this glycoprotein features an array of modules, the most important one being the integrin binding domain. This domain is responsible for cell attachment regulation (11). Other domains are responsible for binding collagen, heparin and various cell surface receptors. Fibronectin fibril formation is not ubiquitous like in the case of collagen or elastin. Instead, additional factors are required for this and it is specific for certain cell types, i.e., fibroblasts. The actin
cytoskeleton plays a major role in fibril formation and integrin transmembrane adhesion proteins are needed to mediate the actin-fibronectin interaction. Besides its architectural role, fibronectin is central for cell migration events.

Laminin is yet another glycoprotein with structural functions. It is most abundant in basal membranes (laminae) and has an asymmetric cross-ressembling structure that allows it to form sheet-like structures further stabilized by disulfide bonds. Laminin commonly associates with a specific type of collagen via certain modulator molecules. Laminin also interacts with the cell surface through integrin receptors and other plasma molecules and regulates cell attachment and differentiation, cell shape and movement, and phenotype maintenance.

Initially perceived as an inert scaffold, ECM was later shown to be involved in cell survival, phenotype and function determination, embryogenesis, morphogenesis, homeostasis, angiogenesis. The importance and role of the ECM was further recognized and emphasized by the emergence of a new field – tissue engineering.

The concept of tissue engineering was extrapolated from the biological observation that dissociated cells can reconstitute in vitro into tissue-like structures, similar to the parental ones, if presented with the appropriate environment. As early as 1933, ECM mimics were used for rudimentary tissue engineering processes. In one model, mouse tumor cells were encased in a polymer membrane, then implanted into chick embryos. Collagen or collagen-GAG composites were later used as scaffolds for skin regeneration.

Biological scaffolds extracted from natural sources (i.e., collagen, fibrin or solubilized basement membrane preparations derived from murine sarcomas) have the
advantage of biological recognition and can undergo cell-triggered proteolytic degradation and remodeling (18). However, cumbersome purification procedures, batch-to-batch variations, immunogenicity and pathogen transmission issues make these materials far from ideal. To address these issues, numerous semisynthetic and synthetic biomaterials have been developed.

1.2. Mimics of natural ECMs

Artificial scaffolds were aimed at recapitulating the key features of their natural counterparts. The ECMs are gel-like, yet they possess an intricate structural design that allows for complex biophysical properties. They are enriched in cell adhesion and recognition sites and harbor an abundance of soluble bioactive effectors such as growth factors. Lastly, the ECMs are enzymatically biodegradable which allow the populating cells to remodel their surrounding accordingly.

1.2.1. Synthetic polymers

Various polymers were employed to reconstitute the intricate mesh formed by the fibrous proteins in the ECM (Table 1.2). Poly(glycolic acid) (PGA) (19, 20), poly(lactic acid) (PLA) (21), poly(lactic-co-glycolic acid) (PLGA) (22), poly(ethylene glycol) (PEG) (23-27), poly(propylene glycol) (PPG) (23), poly(glycolide-co-caprolactone) (PGCL) (28), poly(aldehyde guluronate) (PAG) (29), poly(2-hydroxyethyl (or propyl) methacrylate) (HEMA or HPMA) (30, 31), poly(N-isopropyl acrylamide) (PNIPAAm) (30, 31) are just some of the materials that were used to create composites that can be presented as films, sponges, hydrogels, meshes or fibers. For tissue engineering applications, the most commonly employed synthetic polymers are poly(ethylene oxide)
Table 1.2. Synthetic polymers used as artificial scaffolds.

<table>
<thead>
<tr>
<th>POLYMER</th>
<th>CHEMICAL STRUCTURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA</td>
<td>O</td>
</tr>
<tr>
<td></td>
<td>(-\underbrace{\text{CH}_2\text{--C--O}}_n)\textsuperscript{(n)}</td>
</tr>
<tr>
<td>PLA</td>
<td>(-\underbrace{\text{H\text{--C--C--O}}}_n)\textsuperscript{(n)}</td>
</tr>
<tr>
<td></td>
<td>(-\underbrace{\text{CH}_3\text{--O}}_m)\textsuperscript{(m)}</td>
</tr>
<tr>
<td>PLGA</td>
<td>(-\underbrace{\text{H\text{--C--C--O}}}_n)\textsuperscript{(n)} [(-\underbrace{\text{H\text{--C--C--O}}}_m)\textsuperscript{(m)}]</td>
</tr>
<tr>
<td></td>
<td>(-\underbrace{\text{CH}_3\text{--O}}_m)\textsuperscript{(m)}</td>
</tr>
<tr>
<td>PEG</td>
<td>H(-\underbrace{\text{O--CH}_2--\text{CH}_2\text{--OH}}_n)</td>
</tr>
<tr>
<td>PPG</td>
<td>H(-\underbrace{\text{O--C--CH}_2\text{--OH}}_n)</td>
</tr>
<tr>
<td>PGCL</td>
<td>(-\underbrace{\text{C--CH}_2\text{--O}}_n)\textsuperscript{(n)} [(-\underbrace{\text{C--(CH}_2\text{--O}}_m)\textsuperscript{(m)}]</td>
</tr>
<tr>
<td>PAG</td>
<td>(</td>
</tr>
<tr>
<td>HEMA</td>
<td>(-\underbrace{\text{CH}_2\text{--C--OH}}_n) (-\underbrace{\text{CH}_3\text{--C--OH}}_n)</td>
</tr>
</tbody>
</table>
Table 1.2. (continued)

<table>
<thead>
<tr>
<th>POLYMER</th>
<th>CHEMICAL STRUCTURE</th>
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<tbody>
<tr>
<td>HPMA</td>
<td><img src="image" alt="HPMA structure" /></td>
</tr>
<tr>
<td>PNIPAAm</td>
<td><img src="image" alt="PNIPAAm structure" /></td>
</tr>
<tr>
<td>PEO</td>
<td><img src="image" alt="PEO structure" /></td>
</tr>
<tr>
<td>PVA</td>
<td><img src="image" alt="PVA structure" /></td>
</tr>
<tr>
<td>P(PF-co-EG)</td>
<td><img src="image" alt="P(PF-co-EG) structure" /></td>
</tr>
</tbody>
</table>
(PEO), poly(vinyl alcohol) (PVA) and poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)) (32).

PEO is a hydrophilic polymer, commonly modified at the ends with acrylate or methacrylate groups that allow for photochemical crosslinking (33-35). PEO can also be thermally crosslinked via a reversible reaction, when used as a copolymer with PLA.

PVA can be chemically crosslinked with a variety of compounds (succinyl chloride, adipoyl chloride or glutaraldehyde) (36, 37) to form hydrogels. Moreover, PVA can yield hydrogels by repeated freeze-thaw cycles, when the polymer is in aqueous solutions (38).

P(PF-co-EG) is a synthetic copolymer of poly(propylene fumarate) and PEG that can be crosslinked either chemically or by UV exposure (39, 40). This material was mainly designed and used as an injectable hydrogel for blood vessel engineering and bone repair (41). Synthetic polymer-based biomaterials are discussed in greater detail elsewhere (38-42) and since they are not directly pertinent to the present dissertation, they are just briefly mentioned in this context.

1.2.2. GAG-based synthetic ECMs

Heparin/HS are both sulfated GAGs that consist of repeats of L-iduronic acid (1doA) or D-glucuronic acid (GlcA) and D-glucosamine (GlcNH2) (Table 1.1) (43). The amino group of the glucosamine residue can be acetylated (GlcNAc), sulfated (GlcNSO₃) or unsubstituted. Although considerable confusion exists over the characteristics of heparin versus HS, there are some structural features that allow differentiation between the two. The main distinguishing characteristics are listed in Table 1.3.

The key biological activity of heparin is its anticoagulant activity that arises via the binding of plasma antithrombin III to prevent thrombus formation (1). HS also elicits
antithrombotic activity but to a lesser extent than heparin. Aside from the antithrombotic activity, heparin has high specificity for various growth factors, thus regulating cellular proliferation events and wound healing processes. Due to their unusual biological properties, these two macromolecules were explored for tissue engineering applications (44).

Heparin-chitosan (chitosan is a deacetylated derivative of chitin, a polymer found in the exoskeleton of anthropodes) complexes were investigated for their wound healing processes in in vitro models (45, 46). On a full-thickness wound model, heparin-chitosan gels caused a 90% re-epithelialization, compared to 30% observed for chitosan only treated wounds.

Another heparin complex used was one containing alginate (a polysaccharide isolated from several species of brown algae), crosslinked with ethylene diamine (47). This matrix promoted cellular infiltration and angiogenesis when implanted subcutaneously into rats. Heparin coating (alone or in complex with RGD peptides) of vascular grafts made of poly (carbonate-urea) urethane was also employed in order to improve endothelial cell retention and improve cellular metabolic activity.

Alternatively, chemically modified heparin that contained thiol groups mixed with chemically thiolated HA was crosslinked with polyethylene diacrylate (PEGDA) to yield biocompatible hydrogels for controlled release of growth factors (48-51). In a diabetic mouse wound model, heparin/HA hydrogels containing various amounts of growth factors promoted dramatic wound healing in a dose dependent manner. Hydrogels with 20 µg basic fibroblast growth factor (bFGF) led to 90% wound healing compared to 22% healing observed in the untreated wound (49). In vitro release of growth factors in
Table 1.3. Summary of key features of heparin and HS.

<table>
<thead>
<tr>
<th>HEPARIN</th>
<th>HS</th>
<th>FEATURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-12</td>
<td>10-70</td>
<td>Size (kDa)</td>
</tr>
<tr>
<td>Mast cells</td>
<td>All cells</td>
<td>Biosynthesis</td>
</tr>
<tr>
<td>1.8-2.4</td>
<td>0.8-1.8</td>
<td>Sulfate/hexosamine</td>
</tr>
<tr>
<td>~ 85%</td>
<td>40-60</td>
<td>Glucosamine N-Sulfates (%)</td>
</tr>
<tr>
<td>~ 70%</td>
<td>30-50</td>
<td>IdoA content (%)</td>
</tr>
</tbody>
</table>
hydrogels containing less than 1% w/v heparin was sustained over 42 days and as little as 0.03% w/v heparin reduced the released vascular endothelial growth factor (VEGF) fraction from 30% to 21%, while 3% heparin reduced it to 19% (50). Furthermore, heparin containing hydrogels implanted into the ear pinna of mice maintained and promoted neovascularization over 28 days (50). In addition, these studies showed that high-sulfated structures and low molecular weight heparin are not optimal for crosslinking and hydrogel formation and that less than 25% heparin is desired in these hydrogels. Regardless of the heparin concentration, all hydrogels were cytocompatible. The biodegradability of these hydrogels was around 6 weeks when implanted into rats.

Chondroitin sulfates (CSs) are polysaccharides comprised mainly of N-acetylgalactosamine (GalNAc) and glucuronic acid (GlcA) (Table 1.1) with variable sulfation degrees (2). They are especially abundant in the cartilage, tendons, and cornea. The terminology for this macromolecule changed with time as more structural information became available. Chondroitin-4-sulfate (CS A) is a major placentar component and it is mostly sulfated at carbon 4 of the GalNAc residue. Dermatan sulfate (CS B) is abundant in skin, blood vessels, and lungs. The key feature that distinguishes it from chondroitin A is the epimerization of GlcA to IdoA. Chondroitin-6-sulfate (CS C) is sulfated at carbon 6 of GalNAc and it is primarily found in fish and shark cartilage. Chondroitin-2,6-sulfate (CS D) is sulfated at carbon 2 of GlcA and carbon 6 of GalNAc. The commercially available compound is isolated from shark cartilage. Chondroitin-4,6-sulfate (CS E) was found in squid cartilage. CSs have mainly a structural function conferring compressive resistance to tissues. In the extracellular space, they also interact with numerous proteins and their cell regulatory properties are still under investigation.
CSs may also function as disease markers: elevated CS A levels were associated with chronic periodontitis (52), while the presence of a certain CS containing PG (appican) was linked to Alzheimer’s disease (53).

CS-collagen matrices were obtained via carbodiimide chemistry (54-57). The hydrogels were noncytotoxic and the properties of the biomaterial were significantly improved compared to collagen only. The composite has decreased tensile strength, diminished susceptibility to proteolytic degradation, enhanced cellular proliferation and does not alter the cellular phenotype.

Alternatively, gelatin, the hydrolysis product of collagen, was also chemically crosslinked with CS and used to coat Dacron disks for slow release of antibacterial proteins (58). The presence of CS in the hydrogels allowed for increased loading with lysozyme and a slower release rate. Furthermore, gelatin-CS gels showed satisfactory biocompatibility and nearly complete degradation after 18 weeks postimplantation.

CS-chitosan biomaterials produced through ionic crosslinking were primarily targeted to support chondrogenesis and improve bone repair (59, 60). This composite promoted chondrocyte differentiation and osteoblast migration.

Polymer networks of CS and acrylic acid crosslinked with diethylene glycol dimethacrylate (DEGDA) were attempted for colon-specific drug delivery vehicles (58). The methodology was further improved by synthesizing methacrylate grafted CS that was then crosslinked with acrylic acid. This later formulation ensured a lower sol percentage, higher crosslinking degree and a better retention of CS.

Chemically thiolated CS crosslinked with PEGDA was tested in tympanic membrane repair (61) and wound healing models (62-64). The CS biomaterial led to fast
tympanic membrane closure but caused a marked inflammatory reaction (61). In a rabbit maxillary sinus mucosa wound healing model, CS-containing hydrogels were showed to enhance wound healing at a 4-day end point (62).

HA is the most abundant GAG of the ECM. It consists of nonsulfated repeats of GlcA and GlcNAc (Table 1.1). HA is especially abundant in skin (65), vitreous humour of the eye (65), serum (66, 67) and urine (68). Furthermore, HA was showed to be involved in embryogenesis, morphogenesis, hematopoiesis, cell growth and differentiation. High molecular weight HA is chondroinductive (68) and it is widely used for viscosupplementation in arthritis treatment (69, 70). Low molecular weight HA oligosaccharides have been connected with pathological states as regulators of angiogenesis and migration (71-74). Because of their biocompatibility, numerous HA based biomaterials were formulated and tested for various applications and a few, that will be mentioned here, were or still are commercially available (75).

Esterified versions of HA, generically named HYAFF® were developed by Fidia Advanced Biopolymers, Italy (76, 77). The esterification reaction masks the carboxyl groups of the HA, which makes the final product water insoluble. This permits for these materials to be extruded as membranes or fibers, lyophilized to produce sponges or spray-dried to produce microspheres. These biomaterials degrade well in vivo (110 days) and have good biocompatibility. HYAFF® materials were specifically used as drug-delivery scaffolds. Bulkier molecules such as hydrocortisone or α-methylprednisolone, entrapped or covalently attached, had a satisfactory release rate. However, smaller water-soluble molecules diffused at an undesirably fast rate. Improved versions of HYAFF® were successfully used for intranasal delivery of insulin in sheep or vaginal delivery of flu
vaccine or calcitonin in rodents. Mesh scaffolds (3D HYAFF®) and Laserskin® (HYAFF® membrane) were effectively utilized in tissue engineering for epithelialization (78, 79). HYAFF® 7 version seeded with hepatocytes showed promising results for bioartificial liver devices (80), and cellularized HYAFF® 11 was tested as soft-tissue filler in humans (81). In addition, these materials were successfully used in cartilage and bone repair experiments (82).

GYNECARE INTERGEL®, commercialized by LifeCore, USA, was obtained via ferric hydroxide chelation of HA (83). The hydrogel was intended for adhesion prevention in surgical procedures that normally resulted in bowel obstruction, pelvic pain and infertility. Although promising, this material was withdrawn from the market for causing abnormal inflammatory responses.

Formaldehyde or divinyl sulfone (DVS), were used to crosslink HA and yield Hylan® products (Genzyme Corporation, USA) (84). Hylan® is available in several formulations. Hylan® A is a viscous solution with enhanced rheological properties, commonly used for ophtalmological applications (generic name Hylashield®, Genzyme Biosurgical, USA) (85). Hylan® B is a water-insoluble nonimmunogenic, nontoxic, biocompatible gel, mostly used for viscoaugmentation (86). A different formulation, Hylan G-F 20®, was Food and Drug Administration (FDA) approved for degenerative joint disease and arthritis treatment (87). An antiadhesion Hylan® formulation, Sepragel® (Genzyme Biosurgical, USA) was tested as antiadhesive in surgeries (88).

Restylane® (Q-Med, Sweden), a HA-diepoxide based material, is widely available on the European market for dermal augmentations in plastic surgeries (88). Fidia Advanced Polymers, Italy, also developed an intramolecular crosslinked (esterified) HA-
based viscous hydrogel brand-named ACP™ (auto-crosslinked polymer), targeted for post-surgical adhesion prevention (89). A similarly crosslinked product, INCERT® (Anika Therapeutics, USA), available as gels, sponges or films was developed for the same anti-adhesion market (89).

Seprafilm® (Genzyme Biosurgical, USA), a reasonably successful product, was obtained from HA and carboxymethyl cellulose (CMC), crosslinked with lysine through a carbodiimide mediated reaction. This membrane was FDA approved for postsurgical adhesion prevention (90). Seprafilm® mixed with a polypropylene mesh resulted in a new product, available as Sepramesh® (Genzyme Biosurgical, USA) and used for hernia repair procedures (91).

Alternatively, chemically modified HA was extensively employed for novel biomaterial production with improved cytocompatibility features. The synthesis of thiolated HA derivatives proved to be a very successful avenue for producing biocompatible, cytocompatible, biodegradable, in situ crosslinkable biomaterials for tissue engineering applications. This strategy first involves the modification of the GlcA carboxyl groups with 3, 3'-di(thiopropionyl) bishydrazide (DTP). Then the disulfide bonds are reduced by dithiothreitol (DTT) treatment to yield the thiol modified HA macromolecule (HA-DTPH), which in a final step gets crosslinked oxidatively or chemically to produce gels, films or sponges (92-96). Crosslinked HA-DTPH hydrogels were successfully tested in vitro and in vivo for cytotoxicity and cell adhesion (97).

Alternatively, HA-DTPH was chemically “loaded” with mitomycin-C-acrylamide to serve as a slow-release, drug delivery scaffold for postsurgical adhesion prevention (98, 99). The material was further improved by increasing its carboxyl group content via
treating HA with haloacetic acid. The new carboxymethylated HA (CMHA) was then thiolated (CMHA-S, also known as Carbylan™-S) via the aforementioned procedure. PEGDA crosslinked CMHA-S hydrogels are very promising in numerous surgical applications, including vocal fold repair (96, 100), tympanic membrane repair (61), cortical bone defect repair (101). A different formulation of this hydrogel that contains 50% w/v thiolated gelatin (Gtn-DTPH) was showed to better support cell adhesion and proliferation (available as Extracel™, Glycosan BioSystems, USA). This material is obtainable as hydrogel, film or sponge. Extracel™ is cytocompatible, biodegradable, in-situ crosslinkable under physiologic conditions and performed well in several tissue engineering applications (97, 100-102).

Overall, GAG-based biomaterials represent a very attractive alternative for tissue engineering applications. Their structure allows for a multitude of ways to modify the macromolecule and modulate its biological and biomechanical properties. In addition, GAG-based biomaterials are obtainable as meshed, membranes, films or sponges, which increases their application specific user-friendliness. This dissertation is further intended to present two novel methodologies for HA-based sECM synthesis and discuss several practical applications of these biomaterials.
1.3. References


CHAPTER 2

*IN VITRO* ANALYSIS OF THE EFFECTS OF VARIOUS SYNTHETIC EXTRACELLULAR MATRICES ON FIBROBLAST BEHAVIOR

For this study, CMHA-S and Gtn-DTPH were synthesized by Dr. Xiao-Zheng Shu.

Samples for H&E staining (Section 2.2.5) were processed by Thom Jensen.
2.1. Introduction

Three-dimensional (3D) cell culturing has gained popularity in the past decades, after numerous studies have demonstrated that classical two-dimensional (2D) culturing conditions lead to aberrant cell behavior that is totally irrelevant to the actual in vivo conditions (1-4). In organisms, a complex network of proteins and proteoglycans constitute the extracellular matrix (ECM) that surrounds every cell. Intricate processes such as cell proliferation, migration, differentiation, angiogenesis and invasion are orchestrated by the ECM components and the signaling cascades originated here (5-7). In addition, the ECM dictates the morphology and overall behavior of cells and in turn it is constantly shaped and molded by matrix-specific enzymes produced by cells (8).

ECM scaffolds derived from natural sources address, in some regards, the issues of biological recognition, presentation of receptor-binding ligands, cell-induced proteolytic degradation and remodeling (7). One such material, PureCol™ (Vitrogen®) consisting of 99.9% pure type I collagen, was tested for tissue engineering processes and is widely used as a coating material for medical devices and other applications (9-13). A different ECM facsimile, Matrigel™, is derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma and contains proteins, glycoproteins and growth factors normally found in connective tissues (14). Matrigel™ was successfully utilized for a variety of applications such as cell growth and differentiation, angiogenesis and invasion assays, and was shown to promote a natural cell morphology and behavior (14-18). However, concerns such as limited availability, pathogen transmission or immunogenicity, raised by natural biomaterials, emphasized the necessity of synthetic ECM (sECM) counterparts.
Synthetic analogs of the natural ECMs were developed as 3D scaffolds in an effort to accomplish in vivo-like environments in culture dishes, ex-vivo tissue growth and engineering, and other scientific applications without posing health risks. One such material PuraMatrix™, is synthetic, peptide-based and forms fibrous scaffolds that can be used for 3D cell embedding or surface plating (19-23). Its composition also permits its use for in vivo studies and eliminates the problem of immunogenicity. Another available ECM analog, Extracel™, is semisynthetic and is based on chemically modified hyaluronan (CMHA-S, also known as Carbylan™-S) and gelatin (Gtn-DTPH), crosslinked with acrylate modified polyethylene glycol (PEGDA) (24, 25). This material was effectively tested in numerous tissue engineering applications and is suitable for both 3D and pseudo-3D plating (26-33).

The current study was aimed to evaluate all these materials side by side, for their efficiency as artificial scaffolds and convenience of use. Cell proliferation assays were designed to test the pseudo-3D properties of these materials, while 3D growth and cytotoxicity experiments were included to analyze the effect of the matrices on embedded cells. The results of this study represent a compilation of experimental findings and could be used to rank these sECMs from a researcher’s perspective, taking into consideration the biological performances of the matrices, as well as their preparation protocols and “user-friendliness.”
2.2. Experimental procedures

2.2.1. Preparation of gels and sponges

Matrigel™ (BD, Biosciences Discovery Labware, Two Oak Park, Bedford, MA) gels were made according to the manufacturer’s protocol. Briefly, the material was thawed overnight on ice at 4°C. Subsequently, the product was kept on ice and handled with cold pipettes. After casting, the material was allowed to gel for 10 min at room temperature in the hood before adding medium.

PuraMatrix™ (BD, Biosciences Discovery Labware, Two Oak Park, Bedford, MA) was prepared according to manufacturer’s instructions. The peptide mix was sonicated for 30 min prior use, by using a bath sonicator. Sterile sucrose solution (10% w/v) was used to dilute the material two fold. The protocol instructions were further followed to obtain gels. Briefly, at 5 min after casting, culture medium was gently added on the material to increase the pH of the matrix and promote gelation. The medium was changed three more times in the next 30 min.

PureCol™ (Inamed, Freemont, CA) was mixed with 10X phosphate buffered saline (PBS) as recommended by the supplier and the pH of the solution was adjusted to 7.4 with 1 M NaOH. The prepared collagen solution was then filtered through a 0.45 μm syringe driven filter unit prior casting, to ensure sterility.

CMHA-S/ Gtn-DTPH hydrogels (variations of Extracel™ hydrogels commercialized by Glycosan BioSystems, Inc., Salt Lake City, UT) were obtained by mixing CMHA-S solution (thiol group bearing carboxymethylated hyaluronan) with Gtn-DTPH solution (thiol modified gelatin) and crosslinking this mixture with PEGDA. All components were dissolved in DMEM/F12 + 10% newborn calf serum + 2 mM L-
glutamine + penicillin/streptomycin. The materials were filtered through a 0.45 µm syringe driven filter unit prior mixing to ensure sterility. The hydrogels were cast and allowed to gel in the hood at room temperature before adding medium.

For sponges, CMHA-S/Gtn-DTPH hydrogels, prepared in 1X PBS, pH 7.4 (200 µl/well in 96-well plates) were frozen for 2 h at -80°C and then lyophilized overnight. The sponges were then removed from wells and cut into two identical pieces with a surgical scalpel. Each piece is the equivalent of 100 µl hydrogel. Table 2.1 summarizes the exact composition of each biomaterial and their corresponding annotations.

CMHA-S/PureCol™ gels were prepared by mixing CMHA-S solutions with ready-to-use PureCol™ prepared as described above and crosslinking them with PEGDA (see Table 2.1). CMHA-S and PEGDA were dissolved in 1X PBS, pH 7.4. Gels were allowed to form in the hood at room temperature.

2.2.2. Pseudo 3D cell proliferation assay

The effects of the various sECMs on T31 human tracheal scar fibroblasts (a generous gift from Dr. Susan L. Thibeault, Division of Otolaryngology - Head and Neck Surgery, Department of Surgery and Center for Therapeutic Biomaterials, School of Medicine, University of Utah, Salt Lake City, Utah; Division of Otolaryngology-Head and Neck Surgery, Department of Surgery, University of Wisconsin, Madison) under pseudo-3D plating conditions, were assessed using 96-well plates. Each material tested (50 µl/well) was used to coat one row per plate (a total of seven plates were used, one for each day of the assay). The hydrogels were allowed to gel and were seeded with 3.5 x 10^4 cells/ml. On the third day of culture, the medium was refreshed. Cell numbers were
**Table 2.1.** Description and composition of biomaterials used and their corresponding designations.

<table>
<thead>
<tr>
<th>BIOMATERIAL</th>
<th>DESIGNATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5% CMHA-S/PureCol™ (9/1) + 4% PEGDA 10000 (4/1)</td>
<td>Composite 1 (C1)</td>
</tr>
<tr>
<td>1.5% CMHA-S/3% Gtn-DTPH (2/1) + 4% PEGDA 3400 (4/1) - GEL</td>
<td>Composite 2 (C2)</td>
</tr>
<tr>
<td>0.75% CMHA-S/1.5% Gtn-DTPH (2/1) + 2% PEGDA 3400 (4/1) - GEL</td>
<td>Composite 3 (C3)</td>
</tr>
<tr>
<td>1.5% CMHA-S/3% Gtn-DTPH (2/1) + 4% PEGDA 10000 (4/1) - GEL</td>
<td>Composite 4 (C4)</td>
</tr>
<tr>
<td>0.75% CMHA-S/1.5% Gtn-DTPH (2/1) + 2% PEGDA 10000 (4/1) - GEL</td>
<td>Composite 5 (C5)</td>
</tr>
<tr>
<td>1.5% CMHA-S/3% Gtn-DTPH (2/1) + 4% PEGDA 3400 (4/1) - SPONGE</td>
<td>Composite 6 (C6)</td>
</tr>
<tr>
<td>0.75% CMHA-S/1.5% Gtn-DTPH (2/1) + 2% PEGDA 3400 (4/1) - SPONGE</td>
<td>Composite 7 (C7)</td>
</tr>
<tr>
<td>1.5% CMHA-S/3% Gtn-DTPH (2/1) + 4% PEGDA 10000 (4/1) - SPONGE</td>
<td>Composite 8 (C8)</td>
</tr>
<tr>
<td>0.75% CMHA-S/1.5% Gtn-DTPH (2/1) + 2% PEGDA 10000 (4/1) - SPONGE</td>
<td>Composite 9 (C9)</td>
</tr>
<tr>
<td>2% CMHA-S/ 2% Gtn-DTPH (1/4) + 4% PEGDA 10000 (4/1)</td>
<td>Composite 10 (C10)</td>
</tr>
</tbody>
</table>

% - weight percentage
Ratios - refer to volume ratios
3400 and 10000 - molecular weight in Daltons for PEGDA
monitored each day by using the Cell-Titer 96 Aqueous One Solution Cell Proliferation assay (MTS assay) (Promega, Madison, WI). The A_{490} values, which are directly proportional to the number of viable cells, were plotted against the time course of the assay to yield the growth profile of the cells seeded on various sECMs.

Pseudo-3D growth on sponges was tested by the protocol described above. The various CMHA-S/Gtn-DTPH sponges were prepared by freezing and lyophilizing the gelled materials. T31 human tracheal scar fibroblasts (28, 34) are derived from primary culture and were used for this study because in our opinion, they are representative for type of cells that are normally employed for drug screening or other comparable experiments.

2.2.3. 3D growth assay

To determine the growth of T31 human tracheal scar fibroblasts encapsulated in various gels, cells were entrapped in different material at a final concentration of $10^5$ cells/ml and 100 µl of cell + sECM mix was cast per well of a 24-well plate containing Corning Transwell permeable supports (inserts) with 8.0 μm membrane pore size (Corning Inc., Corning, NY). After gelation, cells were incubated with DMEM/F12 + 10% newborn calf serum + 2 mM L-glutamine + penicillin/streptomycin for 72 h and the MTS assay was used to estimate the number of viable cells in each material.

When sponges were used as growth scaffold, $10^5$ cells/ml were added dropwise on top of each sponge (100 µl/sponge). Cells were allowed to attach for 2 h at 37°C/ 5% CO₂ prior adding additional medium and incubation for 72 h. The procedure described above for gels was then followed.
2.2.4. Live/dead cell assay

The cytocompatibility of sECMs with T31 fibroblasts was evaluated by analyzing cell viability by a double staining procedure that uses calcein AM and ethidium homodimer-1 (EthD-1) (LIVE/DEAD® Viability/Cytotoxicity Kit, Invitrogen, Carlsbad, CA). Calcein AM is a nonfluorescent, cell-permeant molecule that is cleaved inside the cell by intracellular esterases to yield its fluorescent counterpart (green fluorescence). EthD-1 is a nucleic acid stain impermeant to viable cells but can diffuse through the membrane of dead cells where it binds to the DNA and gives a red fluorescence. Cells (3 x 10^5 cells/ml) were encapsulated in various sECMs and cultured for 6 days (the medium was replaced with fresh one on the third day of culturing). The staining solution used was obtained by diluting a stock solution of calcein AM to a final concentration of 2 μM in a 4 μM EthD-1 solution in 1X PBS, pH 7.4. The inserts with gels or sponges were washed three times in 1X PBS, pH 7.4 then incubated for 45 min at room temperature, on a rocker, with the staining solution. After 45 min, the samples were washed again three times in 1X PBS, pH 7.4 then covered with one drop of VectaShield mounting medium for fluorescence (Vector Laboratories, Inc., Burlingame, CA) to prevent bleaching. Cells were analyzed with an Olympus IX70 microscope equipped with Microfire/QCAM CCD (Olympus America Inc., Melville, NY) that had a DAPI and GFP filter, at 100X magnification.

2.2.5. Hematoxylin and eosin (H&E) staining

Same culturing conditions and experimental duration were used as described under Section 2.2.4. The gels and sponges were removed from the inserts and fixed for 5 min in methanol. Samples were then further processed via H&E staining at the LDS
Hospital Electron Microscopy Lab (Salt Lake City, UT). The slides were then analyzed with an Olympus IX70 microscope equipped with Microfire/QCAM CCD (Olympus America Inc., Melville NY) at 400X magnification.

2.2.6. Statistical analysis

Values, represented as mean ± standard deviation (S.D.) were compared using Student’s t-test (2-tailed) with \( p < 0.05 \) considered statistically significant and \( p < 0.005 \) or \( p < 0.001 \) considered highly significant.

2.3. Results

2.3.1. Pseudo-3D proliferation and morphology

The proliferation rates and morphology of fibroblasts cultured on different biomaterials or tissue culture plate (TCP) were first analyzed. The constitution prior to crosslinking of the composites analyzed is summarized in Table 2.2. Two major morphological patterns were observed: stretched, spindle-like shaped fibroblasts or clustered rounded cells. Cells grown on Matrigel™, Composite 1 or Composite 5 adopt a rounded morphology, tend to cluster together and proliferate slower than those grown on surfaces such as TCP (control), PureCol™, PuraMatrix™ or Composites 2-4 (Figure 2.1). These differences in cell behavior were attributed to differences in material composition and density. Looser, less dense materials most probably present fewer adhesion sites for cells forcing them to cluster around the fewer attachment spots available, while denser gels with stiffer surfaces and also poly-lysine coated plastic allow cells to spread out and adopt the classical spindle shape morphology (Figure 2.1). Cell morphologies on Composite 6, Composite 7, Composite 8 and Composite 9 (all sponges) were not
Table 2.2. Summary of the weight percent constitution of composites used.

<table>
<thead>
<tr>
<th>COMPOSITE</th>
<th>% (w/v) COMPOSITION BEFORE CROSSLINKING</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PureCol</td>
</tr>
<tr>
<td>1</td>
<td>0.03</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
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</tbody>
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Figure 2.1. Morphology of T31 human tracheal scar fibroblasts on various sECMs after 5 days in culture. A – tissue culture plate; B – Matrigel™; C PuraMatrix™; D - PureCol™; E - Composite 1; F – Composite 2; G - Composite 3; H – Composite 4; I – Composite 5.
analyzed because of the difficulty in microscopically differentiating cell boundaries from the sponge itself.

Cell proliferation rates on various hydrogels were also different (Figure 2.2). All statistical analyses were reported to the TCP control. Matrigel™, PureCol™ and Composite 1 caused a slower cell division rate when compared to the cells grown on TCP (control) \((p < 0.001)\). Cells grown on PuraMatrix™ had similar behavior with the control group grown on TCP (Figure 2.2, A) \((p > 0.05)\). This is due to the inconvenient preparation protocol that causes an irregular surface coating with PuraMatrix™ so that many cells actually attached to the TCP instead of the biomaterial. Composites 2-5 (hydrogels) influenced cellular proliferation rates based on their density and composition (Composite 2 and Composite 3 are stiffer than Composite 4 or Composite 5, as underlined in Table 2.2) (Figure 2.2, B). Softer gels elicited effects comparable to Matrigel™ \((p < 0.001)\), while stiffer gels, of higher percentage (see Table 2.2) had plastic-like effects \((p < 0.05)\). When Composites 6-9 (sponges) were used to coat wells, fibroblasts proliferated at comparable rates to cells grown on poly-lysine treated plastic \((p \sim 0.05)\) (Figure 2.2, C). The increased proliferation rates on sponges are most probably due to the increased attachment and growth surface provided by the macroporous structures of the sponges.

2.3.2. 3D growth/cell encapsulation

To assess cell proliferation rates under 3D culturing conditions, T31 fibroblasts were encapsulated in different ECMs or seeded onto sponges (Figure 2.3). Three days after seeding, the number of cells in each biomaterial was determined by colorimetric assays.
Figure 2.2. Pseudo-3D proliferation of T31 human tracheal scar fibroblasts on wells coated with a thin layer of biomaterials. Values represented are mean ± S.D., n = 6. A. Cell proliferation rates on TCP (control), PuraMatrix™ (p > 0.05), PureCol™ (p < 0.001), Matrigel™ (p < 0.001) and C1 = Composite 1 (p < 0.001). B. Cell proliferation rates on TCP (control), C2 = Composite 2 (p < 0.05), C3 = Composite 3 (p < 0.001), C4 = Composite 4 (p < 0.001) and C5 = Composite 5 (p < 0.001). C. Cell proliferation rates on TCP (control), C6 = Composite 6 (p > 0.005), C7 = Composite 7 (p < 0.05), C8 = Composite 8 (p > 0.05) and C9 = Composite 9 (p < 0.005). All reported statistical data are relative to TCP on day 7 data points.
Figure 2.2. (continued)
Figure 2.2. (continued)
Figure 2.3. Schematic representation of 3D cell seeding onto sponges and encapsulation into hydrogels (original drawing by Xiaoyu Chen).
The variance for Matrigel™ is high because the viscosity of the material, due to the fast room temperature gelation, causes some pipetting inconsistencies. Therefore, when compared to Matrigel™, the composition of hydrogels appears to have a minimal, statistically nonsignificant influence on cellular properties (p > 0.05 relative to Matrigel™) (Figure 2.4). Nevertheless, softer, looser materials such as Matrigel™, Composite 4 or Composite 5 seem to allow more cellular divisions translating in faster proliferation rates and higher cell numbers. Denser, concentrated materials such as Composite 2 or Composite 3 appear to have decreased cell proliferation rates and the average cell numbers in these hydrogels looked lower that in their low concentration counterparts.

During encapsulation of cells in PuraMatrix™, we encountered similar problems to those in the pseudo-3D assays. Additionally, the encapsulation protocol causes some material loss during the gelation process. Cells cultured in PureCol™ sedimented to the bottom of the inserts because of the prolonged gelation time of the material (45 to 60 min at 37°C) and this condition was not included in the assay because it does not correspond to a true 3D growth environment. Cells seeded on sponges elicited proliferation rates similar to the softer materials (Figure 2.4). Sponges obtained from high concentration hydrogels supported slightly higher proliferation rates (p > 0.05), than the sponges obtained from low concentration hydrogels. This is probably due to the different macroporosity of Composite 6 or Composite 7, that allow for a better penetration during cell seeding.
**Figure 2.4.** Proliferation rates of fibroblasts encapsulated within various ECMs. Light grey bars – cells in hydrogels; black bars – cells seeded onto sponges. Columns represent mean ± S.D., n = 4. C1 = Composite 1, C2 = Composite 2, C3 = Composite 3, C4 = Composite 4, C5 = Composite 5, C6 = Composite 6, C7 = Composite 7, C8 = Composite 8 and C9 = Composite 9 (all p > 0.05 relative to Matrigel).
2.3.3. Cytological analysis

The viability and morphology of encapsulated T31 fibroblasts was determined by a double staining procedure that stains live cells green and dead cells red. Three-dimensionally cultured cells were examined 6 days after seeding. Consistent with the results described above, the compliance of the biomaterial influenced cell morphology and behavior (Figure 2.5). Matrigel™-encapsulated cells appeared to be spread, with fibroblasts eliciting spindle-shaped morphology. Cells embedded in PuraMatrix™ were spindle-shaped, but because of the preparation protocol, most of the cells sedimented to the bottom of the insert and attached to the insert membrane, growing under pseudo-3D conditions. PureCol™ set in 45-60 min at 37°C and this caused similar problems with cells depositing on the insert membrane. When CMHA-S was mixed with PureCol™ in a 9 to 1 volume ratio (Composite 1), cells were three-dimensionally distributed, but were round-shaped. The same morphology was observed in all high concentration, denser materials (Composite 2 or Composite 3). Fibroblasts cultured in Composite 4 or Composite 5 elicited both morphologies – rounded and spindle-shaped. Cell viability in all hydrogels was estimated to be 85-90 % based on the double staining microscopic evaluation. Cells seeded into sponges had spindle-shaped morphologies and were able to form localized networks. However, cell viability in sponges was lower that in hydrogels (50-60 %) and this is most probably due to the seeding procedure that calls for 100 µl cell suspension to be added to sponges and then incubated for 2 h at 37°C prior to medium addition, to allow for cell attachment.

Next, embedded cells were stained with hematoxylin and eosin (H&E) for a more detailed cytological analysis. In addition, this staining method proved to be useful in
Figure 2.5. Live/dead staining of 3D encapsulated cells. A = Matrigel™, B = PuraMatrix™, C = PureCol™, D = Composite 1, E = Composite 2, F = Composite 3, G = Composite 4, H = Composite 5, I = Composite 6, J = Composite 7, K = Composite 8 and L = Composite 9.
visualizing the density of the biomaterials (Figure 2.6). Denser gels (Composites 1 – 3) appear opaque with smooth surfaces due to a tighter crosslinking, while softer hydrogels (Matrigel™, PuraMatrix™, PureCol™, Composites 4 and 5) shred during the cutting process because of looser crosslinking. Fibroblasts embedded in Matrigel™ are organized in clusters that correspond to the structures observed by fluorescent staining. PuraMatrix™ and PureCol™ encapsulated cells appear isolated and concentrated in just a few areas of the hydrogel cross-sections. These observations are consistent with the fact that most of the cells embedded in these materials settled on the bottom of the inserts and grow on the insert membrane.

All hydrogel composites tested allow cell growth and division, which result in frequent clusters of cells. H&E staining was particularly helpful in confirming the differences in pore sizes between Composites 6-9 (Figure 2.6, panels I-L). In all sponges, fibroblasts are anchored to biomaterial convolutions (dark purple loops in Figure 2.6, panels I-L), even bridging between them (Figure 2.6, panels I-L), and are capable of dividing and clustering.

2.3.4. Customized hydrogels

The previous experiments showed a direct correlation between cellular properties (morphology, proliferation) and material density. Although adjusting the stiffness of the composites led to a more Matrigel™-like behavior, the results were not entirely satisfactory. Furthermore, softer gels had extended gelation times, which increased the probability of gravitational cell settling, thus reducing somewhat the desired 3D environment to a pseudo-3D condition. To address these issues, we attempted to find an optimal composite formulation that would allow cells to perform more adequately. By
Figure 2.6. H&E staining of hydrogel embedded cells and cells seeded onto sponges. Cells or cell clusters are indicated with arrows. A = Matrigel™, B = PuraMatrix™, C = PureCol™, D = Composite 1, E = Composite 2, F = Composite 3, G = Composite 4, H = Composite 5, I = Composite 6, J = Composite 7, K = Composite 8 and L = Composite 9.
increasing the biomaterial Gtn-DTPH to CMHA-S ratio (see Table 2.1), cellular attachment and proliferation was enhanced (Figure 2.7). The morphology of the cells was identical to that of cells cultured in Matrigel™ (Figure 2.7, A). In addition, when T31 fibroblasts were encapsulated in Composite 10, cell proliferation/growth rates improved significantly (p < 0.05, relative to Matrigel™) (Figure 2.7, B).

2.4. Discussion

Two-dimensional proliferation assays are useful research tools in providing valuable information regarding cell behavior as a result of genetic manipulation or to evaluate side-by-side various cell lines or types. Analysis of growth factor activity, drug screening, serum batch testing are just a few application that heavily rely on cell proliferation assays (35-38). Commonly, cells are plated on poly-lysine coated tissue culture plates and cell proliferation is assessed over a predetermined period of time. Our experimental results show clear differences in cell morphology and proliferation rates dependent on the nature of the culturing surface. Cells can attach and either spread out or stay clustered, all depending on the composition and nature of the material used for culture plate coating. These radically different patterns should definitely be taken into account when designing or selecting assays, especially for experiments such as drug screening that will be further extrapolated for in vivo testing.

A more physiological alternative would be the use of 3D cultures for all in vitro cellular testing. These culturing conditions are the most intimate in vivo mimics and experimental results would be most pertinent to physiologic situations (4, 39, 40). Cellular growth and morphology is heavily influenced by mechanical stimulation and cues coming from the ECM, as well as soluble autocrine and paracrine regulatory
Figure 2.7. T31 specific customized hydrogel. A. Live/dead staining of fibroblasts embedded in Matrigel™ (left) and the customized hydrogel Composite 10 (right). B. 3D proliferation of fibroblasts encapsulated in Matrigel™ or Composite 10 (p < 0.05). Columns represent mean ± S.D, n = 4.
Figure 2.7. (continued)
signals (7, 41). Matrix composition, stiffness and topography play crucial roles in cellular morphology, behavior, tissue development (42, 43). As concluded from our experimental data, Matrigel™ represents a good choice for 3D culturing, but its use is exclusively limited to in vitro applications (it is murine sarcoma-derived, therefore immunogenic). This ECM is also somewhat inconvenient to use since it gels fast at room temperature, which limits its use to small-scale experimental set-up. PuraMatrix™ is a synthetic, peptide based biomaterial that overcomes the issue of the immunogenicity. However, when compared to the other materials tested in this study, it was the most cumbersome to use material and gave the most inconsistent and unsatisfactory results. The pH of this material is 3.0 and this limits tremendously the time of cell exposure to this environment. Furthermore, the gelation procedure for this material requires extensive handling (medium needs to be changed three times in 30 min, at 10 min intervals). This increases the risk of contaminating cell cultures and, as in the case of Matrigel™, limits its use to small-scale experimental set-up. PureCol™ is collagen based and has long gelation times (45-60 min at 37°C) that make this material unsuitable for 3D applications. However, this material is very “user-friendly” and represents a good candidate for pseudo-3D plate coating. Extracel™ hydrogel variations (Composites 1-10) are hyaluronan and gelatin-based materials, have convenient cross-linking times (15-20 min Composites 1, 2, 4 and 10; 25-30 min for Composites 3 and 5) are suitable for in vitro 3D cell encapsulation and more importantly, were successfully tested in numerous in vivo applications (26-33). These materials, even without the addition of supplementary ECM components or growth factors, are suitable for cell growth and proliferation. As concluded from our results, the Extracel™ materials appear to be the most versatile
sECMs and represent an attractive scaffold for developing customized biomaterials. Compositions could be specifically formulated for individual cell types and applications by incorporation of additional ECM components and growth factors specific for each cell type.

Extracel™ sponges are yet another option for 3D culturing. Our data show Matrigel™-like behavior of cells cultures in these macroporous scaffolds. In addition, these are ready-to-use sECMs, particularly suitable for tissue engineering applications because of their “user-friendliness”. Altogether, the results of this study underline the diversity of the synthetic scaffolds available, their unique properties, and may serve as a guide to selection of the appropriate application specific ECM.

2.5. Conclusion

There is presently a satisfactory variety of biomaterials available for 3D culturing. This provides scientists with good flexibility in choosing the most appropriate sECM for a specific purpose. However, accurate, in vivo-like experimental design should be based on tailoring the existing biomaterials to best mimic the natural cellular environment. Native ECMs are also different in structure and composition based on the cells that secrete them (44). Cell types and the experimental purpose are ultimately the main factors dictating the compliance of biomaterials used for culturing. Individualized 3D environments seem to be the key for best mimicry of physiological conditions. In conclusion, the current study advocates the necessity of revising the current cell culturing procedures and emphasizes the need for customized sECMs that address unambiguous scientific aspects.
2.6. References


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CHAPTER 3

SYNTHESIS OF HYALURONAN HALOACETATES AND BIOLOGY OF NOVEL CROSSTECTOR-FREE SYNTHETIC EXTRACELLULAR MATRIX HYDROGELS

For this study, CMHA-S and Gtn-DTPH were synthesized by Dr. Xiao-Zheng Shu.
3.1. Introduction

Hyaluronan (HA) is a natural nonsulfated glycosaminoglycan, isolated in 1934 from the vitreous of bovine eyes (1). Present in all vertebrates, it is a major component of the extracellular matrix (ECM) (2) and it is involved in vital processes such as cell differentiation (3), angiogenesis (4-7), morphogenesis (8, 9) and wound healing (10, 11). Because of its biological properties, HA has been extensively used in viscosurgery and viscosupplementation (12-14) or as adjuvant for ophthalmologic drug delivery(15).

In addition, HA represents an excellent building scaffold for novel biocompatible and biodegradable polymers. The main reasons for chemical modifications of HA include the modulation of in vivo degradation rates and alteration of the physicochemical properties of the native macromolecule. Chemically-modified HA biomaterials are now used as major components of scaffolds for tissue engineering (16-22), soft and hard tissue repair (16, 23-26), controlled drug release (17, 27) and growth factor release (6, 28-32). Many HA-based biomaterials are terminally modified or “monolithic”(33), that is they lack functionality for further chemical crosslinking under physiological conditions. Of the “living” HA derivatives that allow for further modifications, hydrazide (34-36) and thiol-modified (19, 21, 22, 37, 38) materials feature nucleophilic functionalities that can be crosslinked by polyvalent macromolecular electrophiles. One such biomaterial, HA-ADH, was obtained by modifying HA with adipic dihydrazide (ADH) (39). The pendant hydrazide groups of this macromolecule allow for further alterations such as drug loading (39), molecular probe labeling (40) or crosslinking (41). HA-ADH hydrogels crosslinked with electrophilic poly(ethylene glycol) (PEG) dialdehydes were successfully used for wound healing (34, 35). Thiolated HA biopolymers obtained via a carbodiimide-mediated
synthesis were crosslinked with electrophilic PEG diacrylates (PEGDA) to yield hydrogels for tissue engineering applications (16, 23, 42).

Only the acrylated (43, 44) and methacrylated analogs (45, 46) of HA qualify as electrophilic. For the most part, these (meth)acrylated HA analogs are employed for photoinitiated polymerization. For example, methacrylated HA solutions that were photocrosslinked to yield clear patches were effectively used as sealants for corneal lacerations in rabbit models (46). In a separate study, photocrosslinked glycidyl methacrylate-HA hydrogels were developed for tissue engineering applications (44). Conversely, methacrylated HA was chemically crosslinked with dithiothreitol (DTT) and loaded with erythropoietin and tested for sustained release in vivo and in vitro (45).

We report here the synthesis and characterization of two novel electrophilic derivatives of HA. The new haloacetate derivatives of HA described herein offer "inverted" chemistry that confers reactivity towards with polyvalent macromolecular nucleophiles. The HA bromoacetate (HABA) and HA iodoacetate (HAIA) contain thiol-reactive groups, and thus show an expected mild cytotoxicity in cell culture. However, reacting HABA or HAIA with macromolecular polynucleophiles affords cytocompatible hydrogels. With thiol-modified HA derivatives, hydrogels that prevent cell attachment and spreading can be obtained. However, by including a thiol-modified gelatin derivative as the macromolecular polynucleophile, cytocompatible hydrogels are produced that support cell attachment, spreading and proliferation. Thus, combining electrophilic and nucleophilic HA derivatives can provide novel hydrogels that are intriguing candidates for adhesion prevention and suitable as nonimmunogenic, noninflammatory, and noncytoadhesive coating for medical devices such as stents and surgical implants.
3.2. Experimental procedures

3.2.1. Materials and analytical instrumentation

High molecular weight hyaluronan (HA, MW = 824 kDa) was from Contipro C Co, Czech Republic. Bromoacetic anhydride (BA), hyaluronidase type I-S from bovine testes (HAse, 451 U/mg solid) were from Sigma-Aldrich Chemical Co., Milwaukee, WI. Phosphate buffered saline 10X (PBS), sodium hydroxide (NaOH), hydrochloric acid 12.1 N (HCl), sodium iodide (NaI), dibasic sodium phosphate, heptahydrate (Na₂P0₄·7H₂O) and SpectraPor dialysis tubing MWCO 10.000 were from Fisher Scientific, Hanover Park, IL. SAMSA fluorescein (5-((2-(and-3)-S-acetylmercapto)succinoyl)amino) fluorescein) mixed isomers was purchased from Molecular Probes Inc., Eugene, OR. T31 human tracheal scar fibroblasts were a generous gift from Dr. S. L. Thibeault (Division of Otolaryngology- Head and Neck Surgery, Department of Surgery, University of Utah, Salt Lake City, UT; Division of Otolaryngology – Head and Neck Surgery, Department of Surgery, University of Wisconsin, Madison, WI).

¹H-NMR spectral data were acquired using a Varian INOVA 400 at 400 MHz. UV/VIS spectra and measurements were performed on a Hewlett-Packard 8453 UV-visible spectrometer, Palo Alto, CA. Gel permeation chromatography (GPC) analysis was obtained using the following components: Waters 486 tunable absorbance detector, Waters 410 differential refractometer, Waters 515 HPLC pump and Ultrahydrogel 1000 column (7.8 x 300 mm) (Waters Corp., Milford, MA). The mobile phase for GPC consisted of 0.2 M PBS buffer/methanol (80:20 volume ratio). HA standards used to calibrate the system were from Novozymes Biopolymers, Bågsvaerd, Denmark. An OPTI
Max microplate reader (Molecular Devices, Sunnyvale, CA) was used to determine the 490 nm absorbance values for cell viability assays.

3.2.2. Synthesis of bromoacetate-derivatized hyaluronan (HABA)

Hyaluronan (6.0 g) was dissolved in 600 ml distilled water (1% w/v solution). The solution pH was adjusted to 9.0 by adding 1 M NaOH. Ten equivalents of bromoacetic anhydride were added to the solution and the reaction was stirred for 24 h at 4°C. The reaction mixture was then dialyzed (MWCO 10000) for 3 days against distilled water. The sample was then lyophilized and analyzed. The purity of the sample was determined by $^1$H-NMR and GPC and the degree of substitution (SD) was determined derivatization with SAMSA fluorescein (SD ~ 18%). $^1$H-NMR (D$_2$O); chemical shifts corresponding to the substituent: $\delta = 3.84$ ppm (COCH$_2$Br).

3.2.3. Synthesis of iodoacetate-derivatized hyaluronan (HAIA)

HABA (2.15 g) was dissolved in 215 ml distilled water (1% w/v solution) and reacted with 10 equivalents of NaI. The reaction was stirred overnight at room temperature. Next, the reaction mixture was dialyzed for 3 days (MWCO 10000) and subsequently lyophilized. The purity of the modified hyaluronan was determined by $^1$H-NMR and GPC. The degree of substitution was calculated by SAMSA fluorescein derivatization (SD ~ 19%). $^1$H-NMR (D$_2$O); chemical shifts corresponding to the substituent: $\delta = 3.7$ ppm (COCH$_2$I).

3.2.4. SAMSA fluorescein derivatization

SAMSA fluorescein (25 mg) was dissolved in 2.5 ml 0.1 M NaOH and incubated for 15 min at room temperature. HCl 6 N (35 µl) was then added followed by the addition
of 0.5 ml NaH₂PO₄·H₂O, pH 7.0. HA-BA and HA-IA (5 mg of each) were reacted with activated SAMSA fluorescein (5 equivalents) for 30 min at room temperature. The reaction mixtures were then separated on an Econo-Pac Bio-Rad column (Bio-Rad Laboratories, Hercules, CA) packed with Bio-Gel P-30 Gel with a nominal exclusion limit of 40 kDa (Bio-Rad Laboratories, Hercules, CA) to confirm the covalent attachment. To determine the degree of derivatization for HAHAs, the SAMSA fluorescein – HAHA reaction mixture was dialyzed for 3 days against dH₂O (MWCO 3500) then the A₄₉₄ was spectroscopically determined. The Lambert-Beer equation was then used to calculate the percent of chemical alteration.

3.2.5. HAHA cytotoxicity assay

T31 human tracheal scar fibroblasts were seeded in 96-well plates at a density of 10⁴ cells/ml (100 µl/well) in DMEM/F12 + 10 % newborn calf serum + 2 mM L-glutamine and incubated for 24 h at 37°C/5% CO₂. Stock solutions of 1.5% HABA, HAIA and HA (120 kDa) were prepared in serum free, L-glutamine free growth medium, and the pH of solution was adjusted to 7.5-8 using 0.1 M NaOH. The growth medium was then removed and cells were washed twice with 100 µl of serum free, L-glutamine free medium. Working solutions (100 µl of each 1.5 %, 1 % 0.6%, 0.2% and 0.1% in serum free, L-glutamine free medium) were added onto cells and the plates were further incubated for an additional 24 h. Untreated cells were used as controls. Cell viability was assessed using a previously described biochemical method (47). The assay uses a tetrazolium compound MTS (Cell-Titer 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI) that gets reduced by dehydrogenases of metabolically active (live) cells to yield a colored formazan product. The reduced salt has an
absorbance maximum at 490 nm that can be monitored spectrophotometrically and the intensity of the color is proportional to the number of viable cells in the well.

3.2.6. Gelation studies

Thiol-modified carboxymethylated HA (CMHA-S, also known as Carbylan™-S) (48, 49) was used as the nucleophilic species in the reaction. The working solutions tested for gelation were: 2% w/v CMHA-S, 2% w/v HABA and 2% w/v HAIA solutions in 1X PBS, pH 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0, adjusted by adding 1 M NaOH. The HAHA containing hydrogels were obtained by using a 3:1 nucleophile to electrophile molar ratio. The CMHA-S only hydrogels (control) were crosslinked through disulfide bonds, by exposure to air. The molar ratio used was experimentally determined to be the optimum for hydrogel formation. The solution (flow) to gel (no flow) transition times were determined by the test tube inverting method reported by Jeong et al. (50). A timer was started upon solution mixing, and samples were checked at regular time intervals. If no fluidity was noticeable upon test tube inversion, it was considered that a gel has formed. The experiment was repeated three times, with consistent results.

3.2.7. Nonadherent hydrogels

CMHA-S and HAHA hydrogels of were obtained by dissolving 2% w/v solutions of CMHA-S, HABA and HAIA (1X PBS, pH to 9.0) and mixing them in a 3:1 nucleophile to electrophile molar ratio. The CMHA-S only hydrogels (control) were crosslinked through disulfide bonds, by exposure to air. The composites were then cast in 96 well tissue culture plates and allowed to gel in the hood at room temperature.
3.2.8. Cytoadherent hydrogels

Cytoadherent hydrogels were obtained by adding thiol-modified gelatin (Gtn-DTPH) to the nonadherent hydrogels described above. Briefly, 2% w/v solution of Gtn-DTPH (1X PBS, pH 9.0) was mixed with 2% w/v CMHA-S (9:1 v/v) then reacted with 2% w/v HAHA solutions (1X PBS, pH 9.0) in a 3:1 nucleophile to electrophile molar ratio. The CMHA-S and Gtn-DTPH hydrogels (without HAHA) were crosslinked through disulfide bonds, by exposure to air. As with the nonadherent gels, the composites were cast in 96-well tissue culture plates and allowed to gel in the hood at room temperature. The gelation times for the Gtn-DTPH containing biomaterials were similar to the nonadherent hydrogels.

3.2.9. Hydrogel cytotoxicity assay

Tissue culture plates (96-wells) were coated with 50 μl CMHA-S (oxidatively crosslinked), CMHA-S + HABA, CMHA-S + HAIA, CMHA-S + Gtn-DTPH, CMHA-S + Gtn-DTPH + HABA and CMHA-S + Gtn-DTPH + HAIA hydrogels prepared at pH 9.0 and were allowed to set overnight in hood. Uncoated wells were used as controls. Gels were then washed three times with 200 μl medium (DMEM/F12 + 10% newborn calf serum + 2 mM L-glutamine + penicillin/streptomycin), and then cells (3.5 x 10^4 cells/ml) in the same medium were seeded in each well (100 μl/well). Cells were then incubated for 48 h at 37°C/5% CO₂. The aforementioned colorimetric assay was used to assess the presence of viable cells. Cell attachment was verified microscopically, using an Olympus CKX41 microscope (Olympus America Inc., Melville, NY).
3.2.10. Hydrogel degradation

To determine the rate of enzymatic degradation of hydrogels in the presence of bovine testicular HAse (225 U/ml), 0.5 ml gels were cast in 17 x 60 mm glass vials (Fisher Scientific) and allowed to set overnight. Subsequently, gels were covered with 600 μl 1X PBS, pH 7.4 ± HAse and placed in an incubator at 37°C at 150 rpm. At predetermined time intervals 300 μl PBS ± HAse was removed and $A_{232}$ values were assessed spectrophotometrically (the absorbance range of oligosaccharides is 200-240 nm). For each time point, the supernatant removed for assaying was replaced with fresh one (± HAse, as required). The absorbance value recorded one day after complete digestion was set as 100% and absorbance values read on previous days were extrapolated to percentages.

3.2.11. Statistical analysis

Values, represented as mean ± standard deviation (S.D.) were compared using Student’s $t$-test (2-tailed) with $p < 0.05$ considered statistically significant and $p < 0.005$ considered highly significant.

3.3. Results

3.3.1. Synthesis and characterization of bromoacetate-derivatized HA (HABA)

HABA was obtained by treating a 1% w/v HA solution with 10 equivalents of bromoacetic anhydride (BA) at basic pH (pH = 9) for 24 h (Figure 3.1). The reactant molar excess is needed because of the potential formation of a mixed anhydride between BA and HA, that would rapidly hydrolyze to yield carboxylated HA. This side reaction
Figure 3.1. Synthetic scheme and structure of HABA.
would not cause any interference with the overall biological activity of HABA, however it would limit the availability of primary hydroxyl groups for bromoacetate formation. Next, the reaction mixture was dialyzed for 3 days against nanopure water by using a 10000 MWCO dialysis membrane to remove the bromoacetic acid resulted from the reaction. Subsequently the dialyzed reaction product was lyophilized to give the reaction product (HABA) at 78% yield. The structure of HABA was confirmed by $^1$H-NMR in D$_2$O. Compared to the spectrum of the starting material (HA) (Figure 3.2, A), a new peak appeared at 3.84 ppm corresponding to the methylene protons of the bromoacetate group (COCH$_2$Br) (Figure 3.2, B). The purity and molecular weight distribution of HABA were determined by (GPC). The GPC profile was detected by both UV (Figure 3.3) and refractive index, and confirmed the purity of the compound. The molecular weight of the compound was determined to be MW $\sim$ 120 kDa (polydispersity index 2.58), the decrease in the molecular weight (compared to the starting material) being caused by the acidic reaction side product and the dialysis time. The final HABA product is completely soluble in water. The substitution degree, defined as bromoacetate groups per 100 disaccharide units, was estimated fluorescent dye derivatization to be in the 18% range.

3.3.2. Synthesis and characterization of iodoacetate-derivatized HA (HAIA)

HABA obtained was divided into two equal batches. One batch was used for further chemical and biological characterization. The second batch was used as starting material for HAIA synthesis. A 1% w/v solution of HABA in nanopure water was reacted for 24 h with 10 equivalents of NaI (modified Finkelstein reaction) (Figure 3.4) in a beaker protected from light. The solution was then dialyzed for 3 days against nanopure
Figure 3.2. $^1$H-NMR spectra in D$_2$O. A. HA and B. HABA, showing a new peak at 3.84 ppm, corresponding to the methylene protons of the bromoacetate group (COCH$_2$Br).
Figure 3.2. (continued)
Figure 3.3. GPC analysis (UV) of HABA. The depression in the base line at approximately 21 min is due to the water used to dissolve the sample.
Figure 3.4. Synthetic scheme and structure of HAIA.
water in a 10000 MWCO dialysis membrane and further lyophilized to give the reaction product (HAIA) at 97% yield. $^1$H-NMR was used to confirm the structure of the compound. Compared to the spectrum of the starting material (HABA) (Figure 3.2, B), the peak corresponding to the methylene protons of the haloacetate group ($\text{COCH}_2\text{X}$, $\delta = 3.84$) shifted upfield to 3.70 ppm (Figure 3.5). GPC was employed to assess the purity and molecular weight distribution of HAIA (Figure 3.6). The molecular weight of the compound was determined to be $\text{MW} \sim 160 \text{kDa}$ (polydispersity index 2.45). The substitution degree was presumed to be identical with HABA because of the complete shift of the $^1$H-NMR peak $\delta = 3.84$ to $\delta = 3.70$ and the slight increase in the GPC retention time.

### 3.3.3. SAMSA fluorescein derivatization of HAHAAs

The structures of the two HAHA derivatives were verified by $^1$H-NMR. However, because of the complexity of polymer proton spectra an additional measure was used to test for successful chemical alteration. SAMSA fluorescein is a thiol group containing fluorescent reagent, commonly used for assaying maleimide and iodoacetamide moieties of proteins (Figure 3.7, A). Because of the nature of the novel reactive groups, SAMSAfluorescein derivatization was chosen to assess the presence and reactivity of the new moieties (bromoacetate for HA-BA and iodoacetate for HA-IA). After conjugation of HA derivatives with SAMSA fluorescein as described under Experimental procedures and dialysis, the solutions were photographed under UV light (254 nm) to visually assess the fluorescence intensities (Figure 3.7, B). The covalent attachment of the fluorescent moiety to HAHA was further confirmed chromatographically (Figure 3.8). The results of
Figure 3.5. $^1$H-NMR spectrum in D$_2$O of HAIA. An enhanced peak at 3.7 ppm, corresponding to the iodoacetate group protons (COCH$_2$I) is observable.
Figure 3.6. GPC analysis (UV) of HAIA. The depression in the base line at approximately 21 min is due to the water used to dissolve the sample.
Figure 3.7. SAMSA fluorescein derivatization of HAHAs. A. Chemical structure of SAMSA fluorescein. B. Quantitation of $A_{494}$ absorbance values of SAMSA-derivatized compounds (* $p < 0.001$, versus the HA control). Columns represent mean ± S.D., $n = 3$. Inset – fluorescence intensities of SAMSA derivatized solutions under UV light (254 nm).
Figure 3.7. (continued)
Figure 3.8. The covalent attachment of SAMSA fluorescein to HAHAs, as visualized under UV light (245 nm). **A.** HABA-SAMSA elutes as a separate fluorescent band from the excess SAMSA, indicating that HABA-SAMSA is covalently bonded. **B.** HAIA-SAMSA elutes as a separate fluorescent band from the excess SAMSA, indicating that HAIA-SAMSA is covalently bonded.
Figure 3.8. (continued)
this experiment represent a proof of concept and show the successful chemical alteration of the HA polymer.

3.3.4. HAHA cytotoxicity

T31 human tracheal scar fibroblasts (19, 51) were cultured in 96-well plates and were used as a model system to evaluate the effect of HAHA on cells. The cells were initially cultured in serum containing medium to ensure proper growth. Subsequently, cells were washed with serum free medium, and various concentrations of HAHA (1.5%; 1%; 0.6; 0.2% and 0.1%) in serum free medium were added to cells. Cells covered with serum free medium only were used as controls. After 48 h, cell viability was assessed colorimetrically as described under Experimental procedures.

As expected for highly electrophilic species, the two HAHA polymers were cytotoxic at high concentrations. However, at low concentrations (0.1 % w/v), they were well tolerated by these sensitive cells (Figure 3.9).

3.3.5. HAHA containing hydrogels

To determine the gelation time of biomaterials cross-linked with HAHA, hydrogels were prepared by mixing CMHA-S with HAHA in a 3:1 molar ratio. The pH dependence of gelation times was next investigated. Solution (2% w/v) of CMHA-S and HAHA were made in 1X PBS, pH7.4 and the pH of the solutions was then adjusted to pH 7.0; 8.0; 9.0; 10.0; 11.0 and 12.0. As expected, based on the chemical nature of the haloacetate substituents, the fastest setting solutions were those at pH 9.0 and 10.0 (Table 3.1). The gels obtained were clear and insoluble in aqueous solutions. The gelation process of the HAHA containing hydrogels proceeds via a nucleophilic substitution
Figure 3.9. T31 fibroblast viability in the presence of HAHAAs. Black bar – untreated control; white bars – HABA treated samples; grey bars - HAIA treated samples (* p < 0.005 and ** p < 0.5 *** p > 0.05 versus untreated control). Columns represent mean ± S.D., n = 6.
Table 3.1. pH dependent gelation times for HAHA containing hydrogels.

<table>
<thead>
<tr>
<th>REACTANT</th>
<th>CMHA-S™</th>
<th>GELATION TIME (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromoacetate-HA (HABA)</td>
<td>28</td>
<td>7.5</td>
</tr>
<tr>
<td>Iodoacetate-HA (HAIA)</td>
<td>28</td>
<td>7.5</td>
</tr>
<tr>
<td>CMHA-S™</td>
<td>48</td>
<td>7</td>
</tr>
</tbody>
</table>

| pH | 7   | 8   | 9   | 10  | 11  | 12  |
reaction that leads to the formation of a thioether. The thiol groups of CMHA-S have a pKa value of approximately 9, which explains why the optimum pH of the reaction is 9-10 (38, 48, 49). At lower pH values, the thiol group is mostly in its acidic form, while at pH 9-10 the anionic form is predominant. At pH values above 10, the hydroxyl group displaces I making it unavailable for thioether formation.

3.3.6. Hydrogel cytotoxicity

Hydrogels were prepared by mixing 2% w/v solutions of CMHA-S, pH 9.0 with 2% solutions of HAHA, pH 9.0 in a 3:1 molar ratio. The mixed solutions were then used to coat the wells of a 96-well plate and allowed to gel overnight in the hood. Before cell seeding, the hydrogels were washed serum containing medium then 3.5 x 10^4 cells/ml (100 µl/well) were seeded and incubated at 37°C/5% CO₂ for 48 h.

Previous studies show that HA-based hydrogels such as Carbylan™-SX do not promote cell adherence (16, 23, 52) while HA-based gels that contain gelatin support cell adherence and proliferation (19, 53). To objectively evaluate the cytotoxicity of HAHA containing hydrogels, uncoated wells and wells coated with Gtn-DTPH (gelatin-based material that promote cell attachment) containing gels were used as controls. After 24 h, cells were examined microscopically. Cells seeded on Gtn-DTPH-free materials, were clustered together, rounded and unattached, while cells grown on plastic only or on Gtn-DTPH-containing materials were spread out and elicited the typical spindle-shape morphology. Cellular viability was assessed by the MTS colorimetric assay 48 h after cell seeding. In the absence of Gtn-DTPH cells were not able to attach. Even fewer cells were present on HAHA containing gels, consistent with the cytotoxic effect of these materials (in hydrogels, the final HAHA concentration is 0.67% because a 2% w/v stock solution
is added to the polymer solution at a 1:3 molar ratio) (Figure 3.10). HAHA containing, Gtn-DTPH free hydrogels showed 17% (HABA) to 30% (HAIA) decrease in cell adhesion/viability versus the control hydrogels (CMHA-S only) (see inset from Figure 3.10).

3.3.7. HAHA hydrogel degradation

The use of HAHA hydrogels for medical purposes or any other in vivo application would be dependent on the rate of gel degradation under the action of hyaluronidases which translates to the time that the coating material would actually be present in vivo. To estimate the rate of hydrogel degradation, Gtn-DTPH free hydrogels were incubated with 1X PBS, pH 7.4 ± Hase (225 U/ml). Our results show that CMHA-S hydrogels that are crosslinked via disulfide bonds hydrolyze much faster that the HAHA containing materials (Figure 3.11). By the third day, CMHA-S hydrogels were totally degraded. In contrast, CMHA-S/HABA crosslinked hydrogels appeared totally degraded by day 5, while HAIA containing hydrogels degraded slightly slower (by day 6). In the absence of the enzyme, HAHA containing hydrogels hydrolyzed at a very slow rate. CMHA-S-only hydrolysis rate could not be determined because this biomaterial has a different behavior that the HAHA containing ones and swells upon supernatant addition.

3.4. Discussion

Previously, our laboratory reported the syntheses of chemically modified HA polymers bearing nucleophilic groups that were crosslinked with non-HA electrophilic crosslinkers to yield hydrogels (22, 53). Our approach here was to add elecrophililic
Figure 3.10. Viability of fibroblasts cultured on HAHA hydrogels, as determined by MTS colorimetric assay. Statistical analysis - * $p < 0.001$, ** $p < 0.05$ and *** $p > 0.05$ versus control. Inset – blow-up of A490 values for fibroblasts cultured on Gtn-DTPH-free HAHA hydrogels (* $p < 0.05$ versus CMHA-S). Values represented are mean ± S.D., $n = 6$. 
Figure 3.11. Hydrogel degradation rates in the presence or absence of HAse (225 U/ml). Each data point represents the mean ± S.D., n = 3.
groups onto the HA polymer, namely to introduce reactive bromo- and iodoacetate functionalities onto HA. Our strategy first involved the synthesis of bromoacetate-modified HA (HABA) then this was used to obtain the iodoacetate modified HA (HAIA) via a modified Finkelstein reaction. The direct use of iodoacetic anhydride for HAIA synthesis was avoided because of concerns of compound decomposition in water and the possibility of reaction side products due to the increased reactivity of the reactant. The newly synthesized HAHAs contain thiol reactive groups and when tested in cell cultures (T31 human tracheal scar fibroblasts) they elicited a dose dependent cytotoxic effect. The cells selected for this study – T31 human tracheal scar fibroblasts – were chosen because the use of primary cells is crucial in understanding the biology of normal or diseased tissues. These fragile human fibroblasts are representative for many fibroblastic cells that are normally employed for in vitro biocompatibility and in vitro 3D cytocompatibility experiments.

When reacted with nucleophilic macromolecules, HAHAs formed cytocompatible hydrogels. Depending on the composition, the hydrogels prevent or promote cell adherence, spreading and proliferation. Similar, chemically modified HA hydrogels totally degraded in vitro in 3 days under similar experimental conditions (22). The in vivo residence time of those gels was determined to be more than 2 weeks (16). By analogy, our degradation data indicated that in organisms HAHA containing hydrogels would have a residence time of approximately 4 weeks.

One potential application for the nonadherent HAHA hydrogels could be adhesion prevention. Conditions such as bowel obstruction, pelvic pain, even infertility can be the results of undesired postsurgical adhesions (54). Certain HA hydrogels were already
formulated to address this problem. Drug-loaded hydrogels such as mitomycin-C cross-linked HA hydrogels, were successfully tested for adhesion prevention (51, 55).

Seprafilm®, a carbodiimide-modified HA/carboxymethyl cellulose-based material, has been clinically tested and proven to be successful in reducing adhesion formations after gynecological procedures (56, 57). Carbylan™-SX (PEGDA crosslinked CMHA-S hydrogel) has been shown to be effective in vocal fold repair by preventing scarring and ECM-based dysphonias (27). In addition, this composite was used for postoperative intraabdominal and abdominopelvic adhesions preventions (58). HAHA-based materials could further be used to improve the performances of currently available antiadhesive biomaterials.

Medical device coating is another field that could benefit from the use of HAHA-type biomaterials. Adsorption, ionic coupling, crosslinking, photochemical immobilization, covalent linking or biospecific immobilization are common procedures used for HA coating of medical devices (59). For example, endoluminal metallic stents, used for percutaneous coronary interventions, are commonly coated with biocompatible materials, because of the significant incidence of in-stent restenosis in patients that received noncoated stents (20% to 40% at 6 month after surgical intervention). Carbon, silicon carbide, gold or phosphorylcholine coated stents were previously used for neointimal hyperplasia prevention (60). Drug-coated stents that contained heparin (61) (antithrombotic), dexamethasone (62) (antiinflammatory) or paclitaxel (63) (antiproliferative) were also developed. All these coated materials were targeted to prevent or reduce thrombosis, inflammatory response and aberrant cell adhesion and proliferation. Although theoretically promising, many of the coating materials induced
neointimal hyperplasia leading to restenosis and excessive inflammatory responses months or even years after the surgical intervention (64).

The use of HAHA-based coating materials could provide the awaited solution for restenosis prevention. Unmodified HA was already showed to be adherent to numerous scaffolds (65, 66), therefore HAHAs could be just as easily “attached” to commonly used surgical scaffolds. In addition, the composition of HAHA-based biomaterials would permit the modulation of postsurgical responses such cytoadherence. Their “living” structure would further allow for these materials to be chemically altered and tailored in an application-specific manner.

3.5. Conclusion

Two novel HA derivatives bearing haloacetate groups were successfully synthesized and characterized. Both materials elicit cytotoxic effects in a dose dependent manner when added to regular cell culture medium. When the two new polymers were used to crosslink CMHA-S, a modified HA polymer with reactive thiol groups, the hydrogels obtained did not support cell attachment and still elicited cytotoxic effects. In contrast, the addition of Gtn-DTPH to these hydrogels allowed for cell attachment and growth. This makes these new biomaterials extremely versatile – they can be cytoadhesive or act as barriers to cell attachment, depending on the formulation of the hydrogel. Moreover, HAHA containing hydrogels showed slow HAse-mediated degradation rates, which make them suitable for in vivo applications. Taken together, our results demonstrate the adaptability and potential of the new materials for medical applications, specifically for adhesion prevention and medical device coating.
3.6. References


ERRATUM

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CHAPTER 4

SYNTHESIS AND CHARACTERIZATION OF A NOVEL HYALURONAN DERIVATIVE BEARING THIOETHYL ETHER SUBSTITUENTS

The *in vivo* pilot study described in this chapter was conducted by Dr. Guanghui Yang and Dr. Matthew C. Sardelli.
4.1. Introduction

Hyaluronan (HA) is a naturally occurring unbranched, polyanionic polymer composed of alternating units of glucuronic acid and N-acetylglucosamine (1-3). HA is involved in numerous important processes such as water homeostasis of tissues and joint lubrication (3), cartilage matrix stabilization (3, 4), cell motility (5, 6), morphogenesis and embryogenesis (7), inflammation (8).

Native HA has been extensively used in numerous drug delivery and surgical applications (9), in viscosurgery, viscosupplementation and wound healing (10). Nevertheless, the rapid in vivo degradation of the macromolecule and its unsatisfactory biomechanical properties preclude many clinical applications. Chemical and mechanical robustness were ultimately achieved via chemical alterations and crosslinking strategies. The principal targets of HA for chemical modification are the carboxyl (glucuronic acid residue) and primary hydroxyl (N-acetylglucosamine residue) groups of the molecule. Carboxyl groups have been most commonly modified by esterification via carbodiimide-mediated chemistry (11-15). The highly esterified HA obtained was water insoluble and could be molded into fibers and sponges or turned into microspheres (11). Carbodiimide activated carboxyl groups were also rendered susceptible to nucleophiles such as primary amines (14, 15). The polymers obtained via such carboxy-group derivatization were biocompatible and could further be used for drug-coupling (HA-Mitomycin C) (16, 17), fluorescent probe attachment (HA-BODIPY) (13), hydrogel formation via cross-linking (18, 19) or photopolymerization (20). The hydroxyl groups of HA have been sulfated (21-24), oxidized to dialdehyde functions with periodate (25, 26), esterified (27), etherified (28, 29) or coupled via an isourea intermediate (30). Functionalities such as amides, hydrazines, and thiols were also chemically introduced as pendant groups for
further crosslinking. Most of the customized HA polymers were intended for use as hydrogels (17, 18, 20, 28, 29, 31-33). The improved and controlled enzymatic degradation rates of hydrogels led to their extensive use in tissue engineering applications.

Thiolated HA biomaterials were previously synthesized via carbodiimide activation of carboxyl groups. These strategies yielded self-crosslinkable or crosslinkable polymers that were used to obtain hydrogels and sponges (18, 19). Numerous medical and tissue engineering applications were successfully accomplished by using thiol-modified crosslinked HA composites (15-17, 25-28, 31, 32, 34-37).

Here, we report the synthesis and characterization of a novel hyaluronan derivative that has a minimalist thiol substituent (2-thioethyl ether). The new thiol modified HA polymer designated HASH, is not crosslinkable, most likely due to the low substitution degree and the shortness of thiol chain and steric hindrance that prevents the SH group from reacting with the cross-linker functionalities. This new biomaterial is cytocompatible and appeared to reduce disease progression in an in vivo rat, collagen-induced arthritis model, pilot study. Altogether, our results indicate the potential of HASH as a candidate for arthritis treatment. Further in vivo studies needed to validate its therapeutic/prophylactic roles are currently underway.

4.2. Experimental procedures

4.2.1. Materials and analytical instrumentation

High molecular weight hyaluronan (HA, MW = 824 kDa) was from Contipro C Co, Czech Republic. Ethylene sulfide and 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) were from Aldrich Chemical Co. (Milwaukee, WI). Phosphate buffered saline 10X (PBS), sodium hydroxide (NaOH), hydrochloric acid 12.1 N (HCl), dibasic sodium
phosphate, heptahydrate (Na$_2$PO$_4$·7H$_2$O) and SpectraPor dialysis tubing MWCO 10.000 were from Fisher Scientific (Hanover Park, IL). SAMSA fluorescein (5-((2-(and-3)-S-acetylmercapto)succinoyl)amino) fluorescein) mixed isomers was purchased from Molecular Probes Inc. (Eugene, OR). Dithiothreitol (DTT) was from BioVectra DCL (Charlottetown, PE, Canada). Celite® 545 was from Sigma (St. Louis, MO 63178).

$^1$H-NMR spectral data were acquired using a Varian INOVA 400 at 400 MHz. UV/VIS spectra and measurements were performed on a Hewlett-Packard 8453 UV-visible spectrometer (Palo Alto, CA). Gel permeation chromatography (GPC) analysis was obtained using the following components: Waters 486 tunable absorbance detector, Waters 410 differential refractometer, Waters 515 HPLC pump and UltraHydrogel 1000 column (7.8 x 300 mm) (Milford, MA). The mobile phase for GPC consisted of 0.2 M PBS buffer/methanol (80:20 volume ratio). HA standards used to calibrate the system were from Novozymes Biopolymers, Bågsvaerd, Denmark. An OPTI Max microplate reader (Molecular Devices, Sunnyvale, CA) was used to determine the 490 nm absorbance values for cell viability assays.

4.2.2. Synthesis of 2-thioethyl ether derived hyaluronan (HASH)

Hyaluronan (2 g, MW 824 kDa) was dissolved in 400 ml distilled water (0.5% w/v solution). The pH of the solution was raised to 10.03 with 1 M NaOH. A 5 fold molar excess of ethylene sulfide was added dropwise to the HA solution and the reaction mixture was stirred vigorously overnight in the hood at room temperature. Some precipitation was observed due to ethylene sulfide polymerization. The solution was then filtered on a one inch bed of Celite® 545. To the clear filtrate, a 5-fold molar excess of DTT was added and the pH of the solution was raised to 8.5 with 1M NaOH. The reaction was stirred overnight at room temperature in the hood. After 24 h the pH of the
reaction mixture was decreased to 3.5 with 6 N HCl. The acidified solution was dialyzed (MWCO 10000) against dilute HCl (pH 3.5). Next, the solution was lyophilized and the purity of the sample was determined by $^1$H-NMR (solvent: D$_2$O; chemical shifts corresponding to the substituent: δ = 3.82 ppm (-CH$_2$-CH$_2$-SH) and δ = 3.69 ppm (-CH$_2$-CH$_2$-SH)). Yield = 78%; m = 1.97 g. Degree of thiolation (as determined by DTNB derivatization) = 4.7%. MW = 180 kDa (GPC). Polydispersity index = 1.86.

4.2.3. SAMSA fluorescein derivatization

SAMSA fluorescein (4 mg) was dissolved in 400 µl 0.1 M NaOH and incubated for 15 min at room temperature. HCl 6 N (5.6 µl) was then added, followed by the addition of 80 µl NaH$_2$PO$_4$·H$_2$O, pH 7.0. HA and HA-TEE were each reacted with 5-fold excess of activated SAMSA fluorescein for 30 min at room temperature. The reaction mixtures were then dialyzed (MWCO 2000) against dilute NaOH (pH 9.0) for 3 days. The A$_{494}$ and fluorescence of the SAMSA derivatized compounds were both determined.

4.2.4. HASH derivatization with 4-(hydroxymercuri)benzoic acid sodium salt

HASH (25 mg) was dissolved in nanopure water to give a 1% w/v solution. To this solution 23 mg of 4-(hydroxymercuri)benzoic acid sodium salt was added (approximately 1:1 stoichiometry to disaccharide units) and the reaction was stirred for 24 h at room temperature. The white precipitate formed (unreacted 4-(hydroxymercuri)benzoic acid sodium salt) was filtered out. The derivatized HA was lyophilized and then analyzed by $^1$H-NMR (D$_2$O). New chemical shifts: δ = 7.4 and 7.7 ppm (C$_6$H$_4$).
4.2.5. HASH derivatization with iodoacetic acid sodium salt

HASH (25 mg) was dissolved in nanopure water to give a 1% w/v solution. Sodium iodoacetate (~12 mg) was added to the HA solution and the mixture was stirred for 24 h at room temperature. Subsequent to dialysis against nanopure water (MWCO 3500), the derivatized HASH was lyophilized and analyzed by $^1$H-NMR (D$_2$O). The peaks corresponding to iodoacetic acid protons (ICH$_2$COONa) are in the $\delta = 3$ to $\delta = 3.7$ ppm region.

4.2.6. Thiol-content determination

HASH (24 mg) was dissolved in 8 ml DTNB solution (2 mg/ml in 0.1 M PBS, pH 8.0) and the solution was stirred overnight at room temperature followed by subsequent dialysis for 3 days (Slide-A-Lyzer 10 K dialysis cassette, Pierce, Rockford, IL). The derivatized HASH was then lyophilized and 2 mg of the lyophilized material was then dissolved in 1 ml 0.1 M PBS, pH 7.4. 2.5 ml DTT solution (1% w/w DTT in dH$_2$O, pH 8.5) was added to 0.1 ml TNB-HASH solution. After the mixture turned yellow, the $A_{412}$ was determined using a Hewlett-Packard 8453 UV-visible spectrometer (Palo Alto, CA).

4.2.7. Cytotoxicity assay

T31 human tracheal scar fibroblasts were seeded in a 96-well plate (seeding density was $12.5 \times 10^3$ cells/well in 100 µl) in DMEM/F12 +10% newborn calf serum + 2 mM L-glutamine + penicillin/streptomycin. Cells were allowed to recover and attach for 24 h at 37°C/5% CO$_2$. The next day, the media was replaced with DMEM/F12 containing 1.5%, 1%, 0.6%, 0.2% and 0.1% HA and HASH, respectively. Cells were incubated for an additional 24 h and cell viability in the presence or absence of HASH was assessed.
using a previously described biochemical method (38). The assay uses a tetrazolium compound MTS (Cell-Titer 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI) that gets reduced by dehydrogenases of metabolically active (live) cells to yield a colored formazan product. The reduced salt has an absorbance maximum at 490 nm that can be monitored spectrophotometrically, and the intensity of the color is proportional to the number of viable cells in the well.

4.2.8. In vivo rat arthritis model – pilot study

Wistar rats (6-7 weeks, ~ 400 g) were obtained from Taconic Farms, Germantown, NY. Rheumatoid joint arthritis was induced in rats by injections (one injection at the base of the tail and four to different sites on the back) of bovine type II collagen of 0.1 M acetic acid emulsified in an equal volume of complete Freund’s adjuvant containing 2 mg/ml Mycobacterium tuberculosis H37 RA strain (39). The same treatment was reapplied after 7 days. Two weeks after the first injections, rats were treated with the following intraarticular injections: normal saline (control); dexamethasone (0.2 mg/kg); 2% w/v CMHA-S (thiol-modified carboxymethylated HA), pH 7.5 crosslinked with 4% w/v PEGDA 4:1 polymer to crosslinker ratio; and 3% w/v HASH solution, pH 7.5. All components were dissolved in 1XPBS then sterile filtered through a 0.45 µm syringe driven filter unit. The injection volumes were 0.5 ml/joint, 2 joints for each rat. The joint diameters of the knees were measured using calipers. The measurements were taken before and immediately after injection of the joint and then at 2, 4, 7, 10 and 14 days after joint injection.
4.2.9. Statistical analysis

The data is represented as the means ± standard deviation (S.D.) of number of repeats. Values were compared using Student’s t-test (2-tailed) with \( p < 0.05 \) considered statistically significant and \( p < 0.001 \) considered highly significant.

4.3. Results

4.3.1. Synthesis and characterization of 2-thioethyl ether hyaluronan (HASH)

Thiolated HA derivatives were previously synthesized in our laboratory via carbodiimide chemistry (18, 19, 31, 35, 36, 40-42). This strategy targeted the glucuronic acid (GlcA) residues of GAG disaccharide units. The first step in the procedure involved the reaction of the GlcA carboxyl groups with 3,3'-di(thiopropionyl) bishydrazide (DTP) in the presence of 1-ethyl-3-[3-dimethylamino)propyl]carbodiimide (EDCl). The resulting disulfide bond containing GAGs were subsequently reduced with dithiothreitol (DTT) yielding the thiolated macromolecules. For the synthesis of HASH the approach was to chemically alter the hydroxyl group of the N-acetyl glucosamine (GlcNAc) residues of HA through a reaction with ethylene sulfide, at basic pH (Figure 4.1). Subsequently, the reaction mixture was treated with DTT to reduce any residual disulfide bonds, followed by dialysis and lyophilization. The structure of the new compound was verified by \(^1\text{H}-\text{NMR} \) (Figure 4.2). When compared to \(^1\text{H}-\text{NMR} \) spectrum of HA (Figure 4.2, A), a peak corresponding to the methylene group attached to the former hydroxyl oxygen \((-\text{CH}_2\text{-CH}_2\text{-SH})\), appeared at \( \delta = 3.82 \) ppm. The resonance for the second methylene group, closer to the thiol functionality \((-\text{CH}_2\text{-CH}_2\text{-SH})\) appears at \( \delta = 3.69 \), but is overlapping with proton resonances corresponding to GlcA and GlcNAc protons from the 3-4 ppm region (Figure 4.2, B). The integration of
Figure 4.1. Synthetic scheme and structure of HASH.
Figure 4.2. $^1$H-NMR spectra in D$_2$O. A. HA and B. HASH.
Figure 4.2. (continued)
the methylene proton signals relative to the N-acetyl protons of GlcNAc cannot be used to determine the degree of HA substitution due to the overlapping of the signals. For this, a modified Ellman’s spectroscopic method was employed. The degree of thiolation was determined to be 4-7%. The purity and the molecular weight of HASH (MW ~ 180 kDa) were determined by GPC analysis (Figure 4.3).

4.3.2. Proof of concept

Due to the complexity of the polymer proton ¹H-NMR spectra, additional measures to test successful chemical alteration were employed. SAMSA fluorescein is a thiol group-containing fluorescent reagent, commonly used for assaying maleimide and iodoacetamide moieties of proteins (Figure 4.4, A). Due to the ease of monitoring, this molecule was chosen to assess the presence and reactivity of the SH moieties of HASH. After conjugation of HA and HASH with SAMSA fluorescein and dialysis, the solutions were photographed under UV light (254 nm) to assess the fluorescence intensities (Figure 4.4, inset). The 412 nm absorbance values of the derivatized compounds were examined, showing that addition of the fluorescent dye to the new moieties occurred (Figure 4.4, B). Next, 4-(hydroxymercuri)benzoic acid sodium salt was reacted with flASH (Figure 4.5). This compound was chosen because of its downfield aromatic proton resonance that gives an identifiable signature and the high affinity and specificity of mercury for thiols. Upon reaction completion, and removal of the unreacted, precipitated reagent, the conjugated HASH compound was analyzed by ¹H-NMR (Figure 4.5). The two methylene protons of the thiol substituent (-CH₂-CH₂-SH) shifted upfield to the δ = 3-3.7 ppm region) and the resonances corresponding to the benzoic acid moiety (-C₆H₄-) appeared at δ = 7.4 and δ = 7.7 ppm.
Figure 4.3. GPC analysis (UV) of HASH. The depression in the base line at approximately 21 min is due to the water used to dissolve the sample.
Figure 4.4. SAMSA fluorescein derivatization of HASH. A. Structure of SAMSA fluorescein. B. A494 absorbance of SAMSA fluorescein derivatized HASH (* p < 0.001, versus the HA control). Columns represent mean ± S.D., n = 4. Inset – fluorescence intensities of SAMSA derivatized solutions under UV light (254 nm).
Figure 4.4. (continued)
Figure 4.5. Proof of concept – derivatization of HASH with 4-(hydroxymercury)benzoic acid sodium salt. A. Reaction scheme of HASH with 4-(hydroxymercury) benzoic acid sodium salt. B. $^1$H-NMR spectrum of HASH-mercurybenzoic acid sodium salt complex.
Figure 4.5. (continued)
Lastly, HASH was reacted with sodium iodoacetate (Figure 4.6). As expected, this also resulted in an upfield shift of the methylene protons (-CH$_2$-CH$_2$-SH) (δ = 3.3-3.7 ppm region). Altogether, these reactions confirmed the presence of the thiol modification.

4.3.3. Cytocompatibility of HASH

T31 fibroblasts isolated from human tracheal scar were used to evaluate the cytocompatibility of HASH. These cells are derived from primary culture and were chosen because of their sensitivity. For this assay, the newborn calf serum and L-glutamine were excluded from the media to avoid the potential neutralization of HASH (Figure 4.7). As controls, two different molecular weight HAs were used (MW 120 and 200 kDa). The 120 kDa HA had no cytotoxic effect on fibroblasts regardless of the concentration used. In contrast, the 200 kDa HA was deleterious (p < 0.001) at high concentrations (1.5 - 0.6% w/v) but was well tolerated at low concentrations (0.2-0.1% w/v). Regardless of concentration, HASH had similar effect to the 120 kDa HA on T31 fibroblasts.

4.3.4. Protective role of HASH in a rat arthritis model (pilot study)

Next we analyzed the effect of HASH on disease progression in a rat arthritis model. Dexamethasone, a strong anti-inflammatory drug, was used as positive control in this study. Arthritis was induced in rats by injections containing Mycobacterium tuberculosis. After the disease onset (14 days postinjection), rats were treated with saline only (negative control), dexamethasone, crosslinked CMHA-S (CMHA-SX) or HASH (Figure 4.8). HASH decreased disease progression at a comparable rate with the dexamethasone control. In contrast, CMHA-SX did not appear to affect the disease
Figure 4.6. Proof of concept – derivatization of HASH with iodoacetic acid sodium salt. 
A. Reaction scheme of HASH with sodium iodoacetate. B. $^1$H-NMR spectrum of HASH-sodium acetate.
Figure 4.6. (continued)
Figure 4.7. Proliferation of T31 fibroblasts as determined by MTS assay; A. in the presence of HA 120 and 200 kDa (white bars represent treatments with HA 120 kDa and grey bars represent treatments with HA 200 kDa) and B. in the presence of HASH. Each column represents the mean ± S.D., n= 6. * p < 0.05, ** p < 0.001 and *** p > 0.05 versus the control group.
Figure 4.7. (continued)
Figure 4.8. Effect of various treatments on disease progression in a rat arthritis model. Black – saline treatment; white – dexamethasone treatment; light grey – CMHA-SX treatment; dark grey – HASH treatment. All values represented are ratios of knee joint diameter (mm) on the corresponding treatment day reported to the knee diameter value (mm) of day 0, prior injection (values are referred to as arbitrary units or A.U.). The day 0 data represent knee diameter (mm) right after injection versus knee diameter (mm) prior injection. Columns represent mean ± S.D., n = 2 for saline treatment and n = 4 for all other treatments. For all time points p > 0.05 versus saline; however the control group included just one rat (with, n = 2) versus 2 animals (two injected joints each, n = 4) in each of the other groups.
progression rate. The results were processed as ratios of knee joint diameter (mm) on the corresponding treatment day divided to the knee diameter value (mm) on day 0, prior injection (values are referred to as arbitrary units or A.U.). The day 0 data represent knee diameter (mm) right after injections versus knee diameter (mm) prior injections, for each treatment. Columns represent mean ± S.D., n = 2 for saline treatment and n = 4 for all other treatments. The saline control group included just one animal with the two hind joint injected (n = 2) while the other treatment groups had two rats with two injected hind joints each (n = 4). This accounts for the statistical p > 0.05 values for the treatments.

4.4. Discussion

Arthritis is used to generically refer to over 100 pathological conditions that cause joint pain and inflammation. The two most common diseases responsible for the aforementioned symptoms are osteoarthritis (OA) and rheumatoid arthritis (RA). Osteoarthritis (OA), also known as degenerative arthritis, is caused by the wear and tear of the joints and affects over 20 million people in the United States (43). OA affects particularly large weight-bearing, synovial joints. In contrast, RA is an autoimmune disease that causes inflammation and ultimately results in the destruction of cartilage and bone (44). Anatomically, a synovial joint features a synovial membrane, cartilage, subchondral bone, synovial fluid and a joint capsule. In arthritis, the articular cartilage slowly degrades and ultimately disappears. However, changes also occur in the subchondral bone, the joint capsule and in the synovial fluid. The synovial fluid is mainly comprised of high molecular weight hyaluronic acid (HA). Conversely, in the synovial fluid of OA patients the HA concentration and its molecular weight is lower than normal (45). HA oligosaccharides or HA hexasaccharides were also found to induce nitric oxide synthase leading to increased production of nitric oxide in bovine articular chondrocytes.
(cartilage forming cells) (46). In cultures of human normal adult chondrocytes, HA oligosaccharide treatment led to the loss of proteoglycan (one of the extracellular matrix components) by induction of matrix metalloproteinase 13, through activation of NFκB and p38 MAP kinase (47). Bovine articular chondrocytes were shown to undergo a dose-dependent chondrolysis when treated with HA oligosaccharides (48). All these processes are associated with the progression and aggravation of arthritis.

Viscosupplementation is an intra-articular treatment option for arthritis, that is targeted to restore the physiological viscoelasticity of the synovial fluid (49). Viscosupplementation involves the injection of high molecular weight HA directly into the arthritis affected joint. However, the poor biomechanical properties and fast degradability of the natural HA call for improvements aimed to perfect the properties of this biomaterial.

The experimental data presented here indicate HASH as a potential candidate for arthritis treatment. The material is HA-based, which would provide biocompatibility, is well tolerated by cells and showed promising results in the rat arthritis pilot study. The presence of the SH groups of HASH might act as radical scavengers, thus protecting cells from the damaging effects of reactive oxygen species. Because of the HA scaffold, it can also serve as a joint lubricant, thus encompassing a dual protective function. The macromolecule is not readily crosslinkable via previously employed chemical crosslinking techniques (18, 19, 31, 35, 36, 40-42). However if needed, its structure could further be chemically altered or crosslinked via other crosslinking strategies (i.e., divinyl sulfone or intra-molecular esterification crosslinking) (50, 51).
4.5. Conclusion

We showed here the chemical synthesis and characterization of a novel thiol containing HA derivative. The material obtained is not suitable for hydrogel formation via crosslinking. However, this macromolecule yields viscous solutions when dissolved in water, which makes it suitable for viscosupplementation-type applications. The experimental data points to it being a promising protective agent against inflammatory joint diseases. Further *in vivo* characterization of this novel biomaterial is currently underway. Altogether, HASH or HASH-based products could be important in developing the next generation of OA treatment products.
4.6. References


CHAPTER 5

IN VITRO ANTI-NEOPLASTIC ACTIVITY TESTING OF NOVEL LYSOPHOSPHATIDIC ACID ANALOGS

The lysophosphatidic acid analogs tested in this study were synthesized by Guowei Jiang.
5.1. Introduction

Lysophosphatidic acid (LPA) (Figure 5.1) is the trivial name for 1-acyl-2-sn-glycerol-3-phosphate, a naturally occurring lipid mediator with functions pertaining to cell proliferation, migration and survival (1-5). LPA can be produced intracellularly or extracellularly in response to various growth factors, phorbol esters and LPA itself. While the intracellular LPA is accounted for as an intermediate of the membrane biosynthetic pathway, recent evidence suggests that extracellular LPA is mainly generated by autotaxin (ATX), a member of the ecto-nucleotide pyrophosphatase/phosphodiesterase family. ATX has been associated with processes such as cell motility and tumor invasion, neovascularization, and metastasis, and synthesizes LPA from more complex lysophospholipids such as lysophosphatidylethanolamine (LPC) by hydrolyzing a phosphodiester bond (6-10).

LPA exerts its biological actions via interactions with the LPA receptors that belong to the G-protein-coupled receptor (GPCR) family. Five LPA GPCRs have (8) been identified to date (9, 11-14). LPA1 to LPA3 (previously referred to as endothelial differentiation genes or EDG) have highly conserved amino acid sequences with divergent C-termini. In contrast, GPCR23/P2Y9 and GPCR 92 (corresponding to LPA4 and LPA5) have low structural and sequence similarity with any of the other three LPA receptors (11, 13). The receptors express diverse ligand specificities, meaning that LPA analogs interact with LPA receptor subtypes differently depending on the ligand-receptor affinity. The tissue distribution of the four receptors differs. LPA1 is the most abundant one, whereas the others have a more limited distribution pattern. In cancers, LPA1, LPA2,
Figure 5.1. Chemical structure of LPA.
and LPA₃ are expressed in a tissue and cell line dependent manner suggesting a possible role for LPA receptor expression patterns as cancer markers (8, 13, 15, 16).

Upon ligand-receptor interaction, LPA molecules signal via MAPK, Akt/PKB, Rho, Rac, Ras and other pathways to initiate events such as cell motility, proliferation and survival (1, 17). As mentioned, the pathways activated and the biological effects are dependent on what ligand interacts with which receptor (18). The mechanisms underlying these complex responses are presently poorly understood, mainly because of the lack of specific probes for the receptor types and subtypes.

The physiological functions associated with LPA mediated signaling pathways make LPA receptors very attractive targets for therapeutic intervention. A number of LPA analogs have already been characterized (19-26). The results of these studies represent an encouraging step towards the development of analogs with higher specificity, stability, potency and low or inexistent off-target effects.

The compounds tested in this study were designed to have improved retention of activity and better stability. In the organism, LPA can be degraded enzymatically by phosphatases, acetyltransferases and lysophospholipases (27). Specifically, the phosphomonoester head group can be hydrolyzed by lipid phosphate phosphatases and render LPA inactive. To overcome this, the bridging oxygen was replaced with substituted or unsubstituted methylene groups (phosphonate groups). The phosphonate groups retained their negative charge under physiological condition as this was proven to be crucial for receptor activation (27).

Herein, we investigate the effects of these LPA analogs on three 3D-cultured neoplastic cell lines. The biomaterial used for 3D cultures, Extracel™ (Glycosan
BioSystems, Inc., Salt Lake City, UT), is semi-synthetic and it is based on chemically modified hyaluronan (CMHA-S) and gelatin (Gtn-DTPH), crosslinked with acrylate modified polyethylene glycol (PEGDA) (28, 29). This material was effectively tested in numerous tissue engineering applications and is suitable for both 3D and pseudo-3D plating (30-36).

5.2. Experimental procedures

5.2.1. Materials and analytical instrumentation

Highly invasive cancer cells lines (MDA-MB-231 - breast, SK-OV3 - ovary and CaCO-2 - colon) were from ATCC, Manassas, VA. Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS) and 100X penicillin/streptomycin were from Gibco/Invitrogen, Carlsbad, CA. Cell-Titer 96 Aqueous One Solution Cell Proliferation Assay (MTS colorimetric assay) was from Promega, San Luis Obisbo, CA. Dextran-fluoresceins (MW 10 and 70 kDa) were purchased from Molecular Probes/Invitrogen, Carlsbad, CA and fluorescein isothiocyanate dextran (MW 500 kDa) was from Fiuka/Sigma-Aldrich, St. Louis, MO. Syringe driven filter units (0.45 μm) were from Millipore, Billerica, MA. 24-well Transwell plates with inserts (0.8 μm pore membrane) and 48-well tissue culture plates were from Fisher Scientific Co. (Corning), Pittsburg, PA. Experimental absorbance values were determined with an OPTI Max microplate reader Molecular Devices, Sunnyvale, CA.

5.2.2. Hydrogel preparation

Extracel™ hydrogels were prepared according to the manufacturer’s instructions. Briefly, hydrogels were obtained by mixing 1.5% w/v CMHA-S solution (thiol group
bearing carboxymethylated hyaluronan) with 3% w/v Gtn-DTPH solution (thiol modified gelatin) in a 2:1 volume ratio and then crosslinking this mixture with 4% w/v PEGDA MW 3400 in a 4:1 volume ratio.

All components were dissolved in DMEM + 0.5% FBS + 1X penicillin/streptomycin. Prior to mixing, the pH of the CHMA-S and Gtn-DTPH solution was adjusted to 7.5 with 1 M NaOH. All solutions were filtered through a 0.45 μm syringe driven filter unit prior crosslinking to ensure sterility.

5.2.3. 3D assays

Cells were added to Extracel™ so that after crosslinking a concentration of 2 x 10^5 cells/ml was achieved. In each well of a 24-well Transwell plate with inserts (8.0 μm pore membrane), 100 μl of biomaterial/cell mix was added and allowed to gel. Gelation was observed after approximately 10 min. After the matrix solidified, 1.8 ml media was added to each well and plates were incubated overnight at 37°C/5% CO₂. After 24 h the media was removed and replaced with media containing 10 μM of compounds 1-6 and controls and plates were incubated for an additional 48 hours.

DMEM + 0.5% FBS + 1X penicillin/streptomycin was used throughout the assay to minimize cell proliferation.

5.2.4. Cell viability assays

Cell viability was assessed using a previously described biochemical method (37). The assay uses a tetrazolium compound MTS (Cell-Titer 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI) that gets reduced by dehydrogenases of metabolically active (live) cells to yield a colored formazan product. The reduced salt has
an absorbance maximum at 490 nm that can be monitored spectrophotometrically, and the intensity of the color is proportional to the number of viable cells in the well.

5.2.5. Drug release studies

Hydrogels were prepared as described above and prior gelation fluorescent dextran conjugates (MW 10 kDa; 70 kDa and 500 kDa) were added, to a final concentration of 1.5% w/v. The hydrogels were cast in 0.25 ml aliquots in a 48-well plate. After the gel solidified, 1 ml of 1X PBS pH 7.4, was added to each well and the plate was incubated at 37°C at 120 rpm. At different time points, 100 µl supernatant was removed (quadruplicates for each time point) and the 494 nm absorbance was determined (maximum absorbance wavelength for fluorescent dextran).

5.2.6. Statistical analysis

Values were compared using Student’s t-test (2-tailed) with $p < 0.05$ considered statistically significant and $p < 0.005$ or $p < 0.001$ considered highly significant. Data points represent mean ± standard deviation (S.D.) of the number of experimental repetitions.

5.3. Results

The following compounds were tested: Compound 1 (α-monobrominated, unsaturated LPA analog), Compound 2 (α-monobrominated, saturated LPA analog), Compound 3 (α-hydroxyl, unsaturated LPA analog), Compound 4 (α-hydroxyl, saturated LPA analog), Compound 5 (α-methylene unsaturated LPA analog), Compound 6 (α-methylene saturated LPA analog) and controls: XY19 (25) (ATX inhibitor), LY294002 (phosphoinositide-3 kinase (PI3K) inhibitor) and Taxol (mitotic spindle toxin).
anti-proliferative agent). The structures of the LPA analogs tested are summarized in Table 1. All compounds are water-soluble and solutions were sterile filtered to avoid contamination of cells. The detailed synthetic methods of the aforementioned LPA analogs are described elsewhere (38).

5.3.1. Drug release model

The Extracel™ hydrogel used for 3D culture conditions needed to be tested for its ability to allow unproblematic access of compounds tested to the encapsulated cells. To determine the exclusion limit of the material and whether or not the molecules could penetrate the gel pores and reach the cells, we designed a “reversed” experiment. The rationale was that if compounds of a certain molecular weight trapped inside the hydrogels can diffuse out, the reciprocal would also be true, meaning that molecules of identical size can also penetrate into the gels. As “drug models” fluorescent dextran polymers of known molecular weights (10, 70 and 500 kDa) were used for easy spectrometric monitoring (Figure 5.2). The 10 and 70 kDa fluorescent dyes diffused rapidly from the hydrogel while the 500 kDa dextran remained trapped inside. Based on these results, we concluded that our LPA analogs can reach the hydrogel embedded cells (as illustrated in Table 5.1, all LPA analogs have a molecular weight smaller than 1 kDa).

5.3.2. Breast cancer cells (MDA-MB 231)

Cells encapsulated in hydrogels were treated for 48h with 10 µM of the aforementioned compounds and controls. The assay was designed so that cell proliferation is dramatically reduced (0.5 % FBS) during the assay period.
Table 5.1. Structures and designations of LPA analogs tested.

<table>
<thead>
<tr>
<th>LPA ANALOGS</th>
<th>STRUCTURES</th>
<th>MW (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 1</td>
<td><img src="image1" alt="Structure 1" /></td>
<td>534.2</td>
</tr>
<tr>
<td>Compound 2</td>
<td><img src="image2" alt="Structure 2" /></td>
<td>508.2</td>
</tr>
<tr>
<td>Compound 3</td>
<td><img src="image3" alt="Structure 3" /></td>
<td>472.3</td>
</tr>
<tr>
<td>Compound 4</td>
<td><img src="image4" alt="Structure 4" /></td>
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<tr>
<td>Compound 5</td>
<td><img src="image5" alt="Structure 5" /></td>
<td>456.3</td>
</tr>
<tr>
<td>Compound 6</td>
<td><img src="image6" alt="Structure 6" /></td>
<td>430.2</td>
</tr>
</tbody>
</table>
Figure 5.2. Drug release model for Extracel™ hydrogels showing the diffusion of fluorescent dextran of different molecular weight from hydrogels. Data point represent mean ± S.D., n = 4.
The α-monobrominated Compound 1 and 2 had a no significant effect on cell viability (p > 0.05), while Compounds 3-6 significantly decreased the number of live cells (p < 0.005) (Figure 5.3). Inhibition of ATX by XY19 and inhibition of PI3K also led to decreased cell survival (p < 0.005). The antiproliferative drug Taxol, had no significant effect on the number of live cells (p > 0.5).

5.3.3. Ovarian cancer cells (SK-OV3)

The effect of the LPA analogs on ovarian cancer cells cultured in 3D was next tested. All compounds were added at 10 μM concentration and cells were incubated for 2 days in low serum medium. The cell viability pattern was different from the MDA-MB-231 assay (Figure 5.4). Compounds 1 to 6 substantially decreased the viability (p < 0.005). Similar effects were caused by treatment with LY294002 and Taxol (p < 0.001). Inhibition of ATX activity only slightly decreased the number of live cells (p < 0.05).

5.3.4. Colon cancer cells (CaCO-2)

Next, LPA analogs were included in the low serum tissue culture medium of CaCO-2 cells, at 10 μM concentration. Colon cancer cells responded differently to the treatment than breast or ovarian cancer cell lines (Figure 5.5). The unsaturated, α-hydroxylated Compound 3 was the only one that exerted a statistically significant increase in cell viability (p < 0.001). All the other LPA analogs increased cell viability but to a lesser extent (p < 0.05). Inhibition of PI3K or Taxol treatment resulted in a slight decrease of viable cells (p < 0.05).
Figure 5.3. Effect of LPA analogs on MDA-MB 231 cells. Columns represent mean ± S.D., n = 4 (* p > 0.005; ** p < 0.001).
Figure 5.4. Effect of LPA analogs on SK-OV3 cells. Columns represent mean ± S.D., n = 4 (* p < 0.05; ** p < 0.001).
Figure 5.5. Effect of LPA analogs on CaCO-2 cells. Columns represent mean ± S.D., n = 4 (* p < 0.05 and ** p < 0.001).
5.4. Discussion

LPA receptor stimulation and LPA itself have been associated with cellular proliferation, DNA synthesis, increased protein kinase C activity, intracellular calcium increase, stress fiber formation and cell rounding (4, 39). ATX, the LPA synthesizing enzyme, was also associated with tumor cell growth and motility (8, 10, 39).

The cell lines used in this study are all very aggressive, highly metastatic and invasive. The experimental results among the three types of cell lines widely vary. Nevertheless, these differences in treatment effectiveness are attributable to cell-line specific ATX and LPA receptor expression (Figure 5.6) (8). In MDA-MB 231, at the mRNA level, the predominant LPA receptor is LPA$_1$. LPA$_2$ is also expressed but its levels are approximately four times lower than LPA$_1$. In contrast, the ATX mRNA levels are almost insignificant. In SK-OV3, the ATX mRNA levels are high and all three receptors are expressed. The predominant form is LPA$_2$, followed by LPA$_1$ at comparable levels. LPA$_3$ is expressed in almost insignificant amounts. Different from the other two cell lines, CaCO-2 expresses almost no ATX, and very low levels of LPA$_2$ and LPA$_1$. Based on the aforementioned receptor expression pattern, our results would indicate that Compound 3-6 act as LPA$_1$ antagonists and LPA$_2$ weak agonists. However, receptor-ligand interactions are much more accurately and objectively characterized in systems individually expressing each of the LPA receptors and ATX (38). The $A_{490}$ values of XY19, LY294002 and Taxol treated samples for each cell line reflect the differences in cell proliferation rates and metabolic activity between cell lines. Thus for SK-OV3 cells that are metabolically more active than MDA-MB 231 and CaCO-2 cells, and able to proliferate even at very low serum concentrations (noticeable in the absolute absorbance
Figure 5.6. Messenger RNA levels of ATX and LPA receptors in tumor cell lines (adapted from (8)).
values for each cell line), Taxol decreased the overall A₄₉₀ absorbance values in agreement with its antiproliferative role.

This study is intended to elucidate the therapeutic potential of these analogs, specifically their effects on tumors and tumor progression. Our present experimental data indicate that cell viability, translatable to tumor growth in vivo, could be modulated by using α-substituted LPA analogs, but the results would be tissue specific. However, to accurately characterize the antitumorigenic properties of these LPA analogs, further experiments are needed. Thus, anchorage-independent growth assays in soft agar will be conducted to estimate the LPA analogs effects on colony formation. Invasion and motility assays will be developed to address the influence of LPA analogs on invasiveness and motility. Specifically, scratch wound assays will be used as an initial estimate of cell motility, then modified Boyden chamber assays will be employed to further characterize invasiveness. If needed, a detailed ATX and LPA receptors profiling will be conducted by RT-PCR and protein expression analysis. Ultimately, based on the in vitro experimental outcome, animal studies will be conducted to determine the in vivo effects of LPA analogs on neoplasticity and metastatic potential.

5.5. Conclusion

Our data underline yet again the complexity of cancers and the strenuous path of efficient drug discovery. As mentioned, the LPA analogs generally have a tremendous therapeutic potential, however the use of proper experimental testing is crucial. The results presented in this study point to the need of reanalyzing these compounds for their antiinvasive and antimetastatic potential both in vitro and in vivo. Although in vitro
analysis is the first logical step in drug discovery, animal studies are essential for the elucidation of the pharmacological and therapeutic characteristics of these compounds.
5.6. References


CHAPTER 6

CONCLUSION
6.1. Summary

The work described in this dissertation underlines the importance and value of hyaluronan (HA)-based synthetic extracellular matrices (sECMs) in the light of their applications and presents two novel synthetic methods for the synthesis of HA-based biopolymers. The study described in Chapter 2 highlights the importance of transition from the classical two-dimensional (2D) culturing techniques to the more adequate three-dimensional (3D) methods. Commercially available extracellular matrices (ECMs) were compared side-by-side and evaluated for their user-friendliness and biological and biochemical performances. The experiments designed for this study are representative for research conducted with ECMs. Classical 2D proliferation methods are commonly employed for a variety of screening assays. Our results show that the nature of the growth surface drastically changes proliferation patterns and cell morphology. 3D growth and proliferation are key features for all tissue engineering applications. As shown, the cellular behavior and morphology was widely dependent on the compliance and composition of the extracellular environment. In addition, the key aspects of 2D versus 3D culture are underlined. Experiments conducted in 3D are more pertinent to in vivo conditions, as shown by numerous research groups (1-4), and these results could be readily translatable from the lab bench to clinical application. Altogether, this study reiterates the importance of choosing the proper application-based and cell-specific ECM and culturing technique for research purposes.

Chapter 3 introduces a novel synthetic strategy that led to two new HA-based sECMs – bromoacetate hyaluronan (HABA) and iodoacetate hyaluronan (HAIA). The two compounds were chemically characterized by $^1$H-NMR, gel permeation
chromatography (GPC) and fluorescent dye derivatization. Cell-based assays were conducted to evaluate the effect of the haloacetate HAs on survival, growth and proliferation. Moreover, the study emphasizes the great versatility of these new HA-based biopolymers: depending on the formulation of the biomaterial cell adherence can be widely modulated. Taken together, the data indicate that the haloacetylated HAs could be useful candidates for adhesion prevention and other clinical applications, such as coating of medical devices.

Chapter 4 describes yet another HA-based biomaterial, HASH. Unlike other thiolated HA polymers, HASH has a short, thiol-containing substituent chain and is not crosslinkable. The presence of chemical alteration was investigated and confirmed by three independent methods: fluorescent dye derivatization that allows for easy monitoring, reaction with a mercury containing compound with high specificity for thiols and an additional chemical reaction with a thiol-reactive haloacetate. HASH was successfully tested in cytocompatibility assays. Moreover, in a rat collagen-induced rheumatoid arthritis pilot study, HASH appeared to reduce the disease progression rate. Based on these experimental results, further studies that investigate the protective role of HASH in animal arthritis models are currently underway.

Chapter 5 focuses on one specific aspect of ECM use, namely 3D culturing for drug screening purposes. Six α-substituted analogs of lysophosphatidic acid (LPA) were screened in this study. LPA receptors, LPA and its synthesizing enzyme autotaxin (ATX) have been associated with malignant phenotypes and other aberrant cellular behavior (5-8). The work presented here analyzes the effects of these compounds in 3-D encapsulated cell growth assays. Although a cell-line specific, receptor-expression dependent behavior
pattern was observed, further experiments that concentrate on motility and invasiveness inhibition are required, since effectors associated with these processes are the main targets of LPA and LPA receptors. Overall, the screening 3D technique employed proved satisfactory, allowing the homogeneous diffusion of the tested compound to the encapsulated cells and the LPA analogs tested appear as promising therapeutics.

6.2. Future directions

The work presented here opens numerous avenues to be explored. HA-based biomaterials and composites are widely used in numerous applications and their potential is further being investigated.

As emphasized in Chapter 2, a paradigm shift from the long-used 2D cell culturing method is imperative. As with the natural ECMs that elicit great tissue and cell-specific diversity, sECMs also need to be customized and application-tailored. Composite compliance and composition needs to be addressed in detail and closely matched with the native counterpart. A "one-formulation fits all" approach is not acceptable for research aimed to convert to clinical applications. Individualized matrices, which balance the role and presence of architectural macromolecules with that of cytokines and growth factors, and intimately mimic in vivo environments will need to be developed based on the experimental design and hypothesis. Other primary cell types will need to be tested in the future to completely characterize Extracel™ materials.

Tissue engineering is one of the main markets for 3D matrices. This field has the potential for tremendous scientific and financial rewards. Building anatomical and functional tissues or organs for applications such as transplants, drug testing, plastic and cosmetic surgeries is easily achievable with the proper tools. Cells, growth factors and a
proper scaffold are the main ingredients for success. Close analyses and understanding of the natural interactions and processes will provide the means for fine-tuning the tissue engineering processes. Besides tissue engineering applications, 3D culturing techniques could be employed for "personalized" screens and therapies. For instance, antineoplastic therapies could be customized and individualized to fit the needs of each patient. Specifically, cells isolated from the subject’s tumor could be cultured and tested for drug effectiveness, efficiency or resistance. Combination therapies could also be designed and tested for synergistic or counteracting effects of drugs. The study described in Chapter 5 represents a step in this direction. As indicated, the LPA analogs appear promising as antineoplastic, therapeutic agents. Further, these compounds will need to be tested in \textit{in vitro} anchorage independent growth, invasion and migration assays. Animal studies conducted to address the effect of the LPA analogs on tumorigenicity, metastatic potential and angiogenesis are also needed. We anticipate that these compounds will prove to be antimetastatic and anti-invasive and that they will translate into clinical practice as components of combination therapies targeted to malignant tumors.

The use of biomaterials is not limited to tissue and cell culturing applications. As mentioned in Chapters 3 and 4, biocompatible HA-based chemically modified polymers could be used for various applications such as viscosupplementation and viscosurgery, having improved biomechanical and biological properties compared to the unmodified HA. The haloacetate HA biomaterials will be used in the future to coat Dacron disks and evaluate the \textit{in vitro} endothelial and smooth muscle cell growth on these surfaces. Based on the experimental outcome of these experiments, \textit{in vivo} studies with HAHA coated
stents will further be designed to address the ability of these materials to prevent restenosis.

The properties of HASH also require further investigation. Our preliminary results show that HASH reduces arthritis progression in rats. In vitro experiments targeted to address the HASH-mediated protection mechanism on chondrocytes are needed. Our hypothesis is that the thiol groups of HASH act as radical scavengers and protect chondrocytes from oxidative damage. One way to test this hypothesis is to grow chondrocytes three-dimensionally in HASH containing hydrogels, challenge them with various reactive oxygen species (i.e., H₂O₂ and sodium nitroprusside) then assess cell viability, proliferation, nuclear fragmentation as a measure of apoptotic rate, etc.

Overall, haloacetate HA and HASH have the capacity of being further modified and tailored to fit every need. Drugs, growth factors or other desired molecules could be covalently attached or simply entrapped in these materials. The crosslinking alternatives are also various and allow for the molding of scaffold properties, such as cytoadherence, biodegradability, crosslinking time, pore size, etc. All these unique properties of HA-based biomaterials open numerous research avenues to be investigated.
6.3. References


