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Engineered Micro-Environments and Vibrational Culture Systems for Vocal Fold Tissue Engineering

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ENGINEERED MICRO-ENVIRONMENTS AND VIBRATIONAL CULTURE
SYSTEMS FOR VOCAL FOLD TISSUE ENGINEERING

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

by
Jaishankar K Kutty
August 2008

Accepted by:
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ABSTRACT

Voice is produced by the conversion of aerodynamic energy from exhalation to acoustical energy for voice production by the vocal folds (membranous connective tissue) located in the larynx. The quality of voice depends on the biomechanical properties of the multi-layered vocal fold tissue which derive from its extracellular matrix (ECM) organization and composition. The wound healing response to vocal fold injuries is characterized by scarring and subsequent dysphonia due to alterations in the biomechanical properties of the tissue.

The work presented here is motivated by the importance of voice in maintaining quality of life and the inability of current treatment techniques to restore long-term, normal phonatory voice following injury induced scarring. We hypothesize that vibration is the epigenetic stimulus regulating the unique extracellular matrix (ECM) composition of the human vocal fold tissue and that rapid restoration of the vibratory microenvironment using mechano-mimetic scaffolds will facilitate:- (a) inhibition of scarring and (b) stimulation of fibroblast regeneration of the normal vocal fold tissue architecture/ECM composition and thereby restore long-term, normal phonaory voice. Our objective was to create in situ photopolymerizable, degradable, mechano-mimetic hydrogels/semi-interpenetrating networks (semi-IPNs) which may be introduced into critical size vocal fold defects using minimally invasive methods.

Towards this end, we created hyaluronic acid (HA) based hydrogels and polyethylene glycol (PEG)-diacrylate based hydrogels/semi-IPNs, which were found to approximate the viscoelastic mechanical properties of the native human vocal mucosa

(vibratory component) and the vocal ligament (strain component), respectively. Cell culture studies indicated that these hydrogel/semi-IPN materials supported cell spreading, cell proliferation, and ECM deposition throughout the 3-dimensional crosslinked network. In an attempt to assess the ability of the HA-based hydrogels to support human fibroblast formation of vocal mucosa-specific matrix in response to physiologically relevant high frequency vibration, fibroblast encapsulated hydrogels were subjected to 2 hrs of vibration per day using a custom built vibrational bioreactor. Our results indicated that the exposure of HA hydrogel-encapsulated fibroblasts to physiologically relevant high frequency vibration stimulated a pattern of gene expression and ECM synthesis (upregulation of GAGs, downregulation of fibrous matrix proteins) consistent with the composition of the human vocal mucosa. In the future, these HA-based hydrogels introduced into the human vocal mucosa during the acute phase of wound healing by minimally invasive methods may bring about regeneration of the native human vocal mucosa ECM composition and thereby restore normal phonation.

DEDICATION

This work is dedicated to Shashikala Krishnan, my mother and Kutty Krishnan, my father, for being tremendous sources of knowledge, inspiration, support, and encouragement throughout the years. Amma (mother) and Achha (father) – inspite of the unthinkable physical distance, you have always been just a phone call away! Thank you for guiding me back on track when I became distracted by other pursuits. You have been my sounding board, therapists, and unflappable companions through the highs and lows. Thank you for keeping me grounded and making me what I am.

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CHAPTER ONE

INTRODUCTION

Voice is critical to the quality of life and is the primary ‘tool of trade’ for 25% of the US work force (teachers, singers, sales personnel etc) [1]. Phonation (voice production) is a co-ordinated biological process involving the vocal folds, lungs, brain, peripheral nervous system, and muscles of the respiratory tract synergistically transducing aerodynamic energy from exhalation to acoustic energy. The vocal folds are the dynamic mechanical element of the phonatory system and possess a spatially organized ECM composition that is fundamental to the tissue biomechanics and in turn the quality of voice [2]. The highly differentiated human vocal fold tissue is comprised of a matrix rich lamina propria anchored to the vocalis muscle, and covered by a squamous epithelium. The fibroblast is the primary matrix producing cell type in the vocal fold tissue and is responsible for maintaining tissue homeostasis and regulating injury response [2]. The squamous epithelium and the superficial lamina propria together form the *vocal mucosa* (vibratory component) which is abundant in hyaluronic acid, and proteoglycans like fibromodulin, decorin and versican which regulate the water content of the tissue and impart viscous shear properties to it [3, 4]. The vocal mucosa vibrates at frequencies between 100 – 1000Hz, and amplitudes of ~1mm [5]. The intermediate and deep layers of the lamina propria together form the *vocal ligament* (strain component). The vocal ligament ECM is comprised mainly of elastin fibers which impart flexibility to the tissue and collagen fibers which impart resistance to tensile stresses [6, 7]. The

human vocal ligament operates at low strain levels (0 – 15%) during normal phonation [8]. Vocalization times can vary between 1 to 2hrs per day for heavy voice users like classroom teachers and opera singers [9-12].

Vocal fold damage can be brought about by a multitude of factors like chronic vocal overuse, chemical/thermal trauma, non phonatory mechanical trauma such as surgical intubation and surgical treatment of infectious laryngeal diseases, laryngeal cancer, and benign pathologies like vocal folds polyps, nodules and edema [13-15]. Injury of the lamina propria elicits a wound healing response that is characterized by the replacement of native parenchymal matrix with fibrous scar tissue. This change in the ECM composition leads to alterations in the tissue biomechanics which subsequently leads to dysphonia. Approximately 23% of the voice dependent USA workforce is afflicted by problems like increased effort to sustain phonation, decrease in the quality of voice and total loss of audible voice as a result of vocal pathologies [16]. Vocal pathologies can jeopardize social interaction, job security, and employment opportunities. Dysphonia secondary to vocal pathologies is a result of hoarseness, breathy voice, stridor (high pitched, loud respiration), and dyspnea. Incidence of voice related problems is as high as 20% amongst teachers [17].

Treatment of scarring related voice problems is a major challenge faced by otolaryngologists today [18]. Voice therapy combined with the administration of steroids is the first line of therapy when presented with vocal fold defects and scars [13, 18]. However, voice therapy is ineffective when presented with scars in combination with pathologies like vocal polyps, nodules and edema despite rigorous adherence to the

prescribed habits [19]. Synthetic, non degradable materials like teflon [20-22], polyhydroxyethylmethacrylate (pHEMA) hydrogels [23], ePTFE [24, 25], calcium hydroxyapatite [26], dacron [27], polydimethylsiloxane [28-31] have been injected into the vocalis muscle in an attempt to overcome scarring-induced glottic incompetence through vocal fold medialization. However, physiological limitations such as migration from the site of implantation, foreign body granuloma formation, and fibrous encapsulation posed by these materials, and the theoretical advantages of natural, degradable biomaterials injected into the lamina propria have led to the use of materials like collagen, fat, fascia and HA for vocal fold augmentation purposes. In human subjects, though evaluations after six months of collagen injections indicated improvements in vibrational amplitude of the mucosal wave and amelioration of glottic incompetence, no perceptual improvement (acoustic analyses) was recorded [32]. Autologous fat [33-36] and fascia [37] injections yielded positive results short term but reports of these studies are inconsistent in terms of evaluations of glottic closure, amplitude of the mucosal wave and the effect on the stiffness of scar tissue. Injectable crosslinked HA gels (Hylan B gels) have been shown to improve mucosal wave amplitudes and glottic closure for approximately 12 months [38-40]. Material resorption and the need for re-injection were the disadvantages associated with the use of natural materials like collagen, fat, fascia, and HA [41]. Current regeneration strategies have investigated the use of crosslinked composites of polyethylene glycol diacrylate (PEGDA)/derivatized HA [42, 43], composite collagen hydrogels [44], and HA-based microgels [45] as scaffolds for cell transplantation and matrix formation, as well as the

administration of hepatocyte growth factor (HGF) [46-48] and basic fibroblast growth factor (bFGF) [49] to reduce scarring during natural healing. The above mentioned current treatment techniques, although effective in improving short term voice quality, have been unable to achieve complete restoration of voice due to their inability to bring about regeneration of the native vocal fold tissue architecture/ECM composition.

Matrix composition and mechanical properties of human tissues are influenced by their mechanical environment [50, 51]. Physiologically relevant mechanical loading not only upregulates synthesis of tissue-specific ECM proteins but also plays a vital role in matrix modeling (native tissue homeostasis) or remodeling (tissue engineering scaffolds) and cellular organization [51]. Recently, Min and co-workers reported that physiologically relevant compressive loading of chondrocyte seeded, mechano-mimetic poly (l-lactide-co- ϵ -caprolactone) scaffolds upregulated the synthesis of cartilage specific ECM proteins like collagen II and proteoglycans like aggrecan [52]. Studies performed by Hsieh and co-workers indicated that mRNA levels of collagens type I and III produced by anterior cruciate ligament (ACL) and medial collateral ligament (MCL) fibroblasts showed significant differences in after physiologically relevant biaxial stretching [53]. Durante and co-workers reported an increase in collagen I synthesis by smooth muscle cells in response to 10% cyclic strain [54]. The effect of cyclic strain on gene/protein expression in cell seeded substrates is widely investigated but that of physiologically relevant high frequency vibration is relatively less explored. Studies reported previously by Tanaka and co-workers indicated that the effects of vibration on in vitro cell culture are varied. For example, mRNA expression levels of MMP-9 were

upregulated when osteoblasts were cultured in 3D collagen gels under conditions of broad frequency vibration (0-50Hz) 3min/day for 4 to 7 days [55]. Contrastingly, the mRNA expression levels of MMP-9 were unaffected by a single frequency (3Hz) vibration 3min/day for 4 to 7 days. Titze and co-workers [11] highlighted the importance of the vibratory epigenetic stimulus in the vocal fold tissue by indicating a similarity between the normal matrix composition of the human vocal mucosa and the gene expression levels of fibroblasts cultured under vibratory stimulation within 3D, porous, elastomeric tecoflex substrates. Thus one may infer that the type of mechanical regimen dictates alterations in protein expression which ultimately influences matrix remodeling.

We therefore hypothesize that the rapid restoration of the vibratory microenvironment using mechano-mimetic scaffolds will facilitate vocal fold-like ECM deposition by the transplanted/endogenous cells. This will potentially yield improved functional outcomes by preventing the onset of fibrosis during the acute stage of wound healing. Towards this end, the objective of our research was:- (a) to create injectable, photopolymerizable, hydrogel formulations matching the viscous and elastic mechanical properties of the vocal mucosa and vocal ligament, respectively, (b) to investigate the cytocompatibility of the potential scaffold materials in terms of encapsulated cell viability, proliferation, and de novo ECM synthesis under static conditions, and (c) to assess the ability of the methacrylated HA (GMHA) based hydrogels to support human fibroblast formation of vocal mucosa-specific matrix in response to physiologically relevant high frequency vibration using a custom-built vibrational bioreactor.

CHAPTER TWO

LITERATURE REVIEW

Vocal folds are the same as **vocal cords**. The two terms refer to the exact same part of the body performing the exact same functions. Scientists and otolaryngologists refer to this organ as “*vocal folds*” whereas “*vocal cords*” is a widely used lay term. The term “*vocal cords*” lacks technical accuracy. Many years ago the *vocal folds* were thought of as being two cords stretched across the airway in the trachea akin to the strings of a musical instrument. In 1974, pioneering research by Hirano led to the understanding that the vocal folds are multilayered folds of tissue that are continuous with other tissues in the laryngeal framework [56]. Therefore, **vocal "folds"** is a more accurate term.

2.1 Anatomy of the vocal folds & physiology of voice production

2.1.1 Anatomy of the larynx

The larynx is located anteriorly in the human neck immediately below the pharyngeal division which gives rise to the trachea (windpipe) and the gullet (food pipe). Figure 2.1 illustrates the longitudinal section of the human larynx. The larynx comprises the cricoid cartilage (smaller), thyroid cartilage (larger), arytenoid cartilages, vocal folds, glottis, epiglottis and the hyoid bone. In males the prominent anterior bulge of the thyroid cartilage is known as the *Adam's apple*. The vocal fold tissue is located antero-posteriorly in the center of the laryngeal framework. Figure 2.2 indicates that the vocal

fold tissue in an intermediate position is a symmetrical “V” shaped structure when viewed from the top. Posteriorly the vocal folds are attached to the arytenoid cartilage.

The vocal folds tissue is ~11 – 15mm long in females and ~17 – 21mm in males [57, 58]. Below the age of 10 there is no difference in the length of the vocal folds between men and women [57]. The membranous vocal fold tissue is ~8.5 – 12 mm long in females and ~14.5 – 18mm in males [57, 58]. In newborns the length of the membranous vocal fold is 1.3 – 2.0mm [57]. The cartilaginous vocal fold tissue is ~2.0 – 3.0mm long in females and 2.5 – 3.5mm long in males [57]. In newborns the length of the cartilaginous vocal fold is 1.0 – 1.4mm [57].

The space between the vocal folds is known as the glottis. The glottis opens when the vocal folds adduct and closes when the vocal folds abduct i.e. vibration causes high frequency glottal opening and closure. The epiglottis (soft cartilage) which lies superior to the vocal folds serves to protect the larynx during deglutition (swallowing) and prevents aspiration of food particles. It is not involved in normal voice production. The hyoid bone positioned above the thyroid cartilage provides an attachment site for many muscles of the tongue, jaw and neck. It is the only bone that is not connected to another bone in the body.

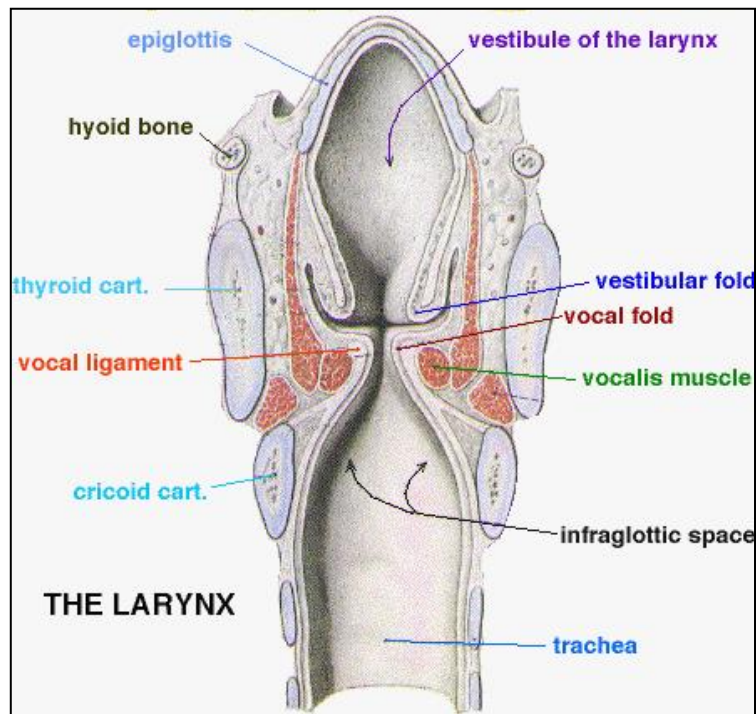


Figure 2.1 Longitudinal section of the human larynx which houses the vocal folds

(<http://www.hopkinsmedicine.org/voice/anatomy.html>)

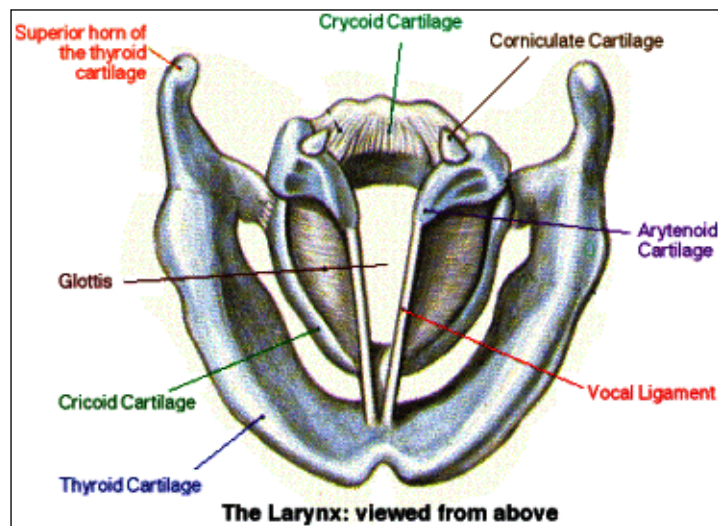


Figure 2.2 Top view of the human vocal folds housed in the laryngeal framework

(<http://www.hopkinsmedicine.org/voice/anatomy.html>)

2.1.2 Laryngeal muscles

There are two types of laryngeal muscles namely, **intrinsic** and **extrinsic**. The intrinsic and extrinsic laryngeal muscles together open and close the vocal folds as well as shape the vocal tract during phonation.

(a) **Intrinsic laryngeal muscles** - These muscles serve to position the vocal folds during the phonatory cycle. They are paired and symmetrically arranged on both sides of the larynx. The five intrinsic laryngeal muscles and their functions are as follows:-

- **Posterior cricoarytenoids** - These are the only muscles involved in abduction (“abduction” - process of pulling the vocal folds away from each other or vocal fold opening). These muscles achieve glottal opening by pulling the back ends of the arytenoid cartilages together. This pulls the front ends (where the vocal folds attach) apart, thus pulling the vocal folds apart.
- **Lateral cricoarytenoids** - These muscles are involved in adduction (“adduction” - process of medialization of the vocal folds or vocal fold closure). These muscles achieve glottal closure by pulling the back ends of the arytenoid cartilages apart. This pulls the front ends together, thus medializing the vocal folds.
- **Thyroarytenoids** - These muscles form the “body” of the vocal folds. They are also known as the “vocalis muscle”. These muscles bring about vocal fold shortening by pulling the arytenoid (back) end of the vocal folds toward the thyroid (front) end. This manner of shortening causes the vocal folds to vibrate more slowly thus lowering the frequency (pitch). The thyroarytenoid muscles provide force to strengthen glottic closure

in an attempt to resist the exhaled airstream from the lungs in order to buildup subglottic air pressure to initiate phonation.

- **Cricothyroid** - These muscle are antagonists to the thyroarytenoid muscles. The cricothyroid muscles serve to lengthen the vocal folds. They lengthen the vocal folds by pulling the thyroid cartilage down, which increases the distance between the arytenoid and the Adam's apple. This causes the vocal folds to vibrate faster, thus raising frequency (pitch).
- **Interarytenoid** - The transverse and oblique arytenoid are the two sets of arytenoid muscles. These muscles bring the two arytenoid cartilages together to provide compression medialization for the vocal folds which along with the forces provided by the lateral cricoarytenoid and thyroarytenoid muscles allow buildup of the subglottic pressure necessary for the onset of phonation.

(b) **Extrinsic laryngeal muscles** - These muscles are also known as the “strap” muscles since they are responsible for moving the entire larynx up and down during respiration, phonation and deglutition. The extrinsic laryngeal muscles can be classified as the infrahyoid and the suprahyoid muscles. The infrahyoid muscles comprise omohyoid, sternohyoid, sternothyroid and thyrohyoid muscles. The suprahyoid muscles comprise digastric, geniohyoid, myohyoid, stylohyoid and stylopharyngeus muscles.

2.1.3 Laryngeal innervation

The vagus nerve which arises from the medulla oblongata in the brainstem provides control over the intrinsic laryngeal muscles. The vagus nerve branches into the

superior laryngeal nerve and the recurrent laryngeal nerve. The superior laryngeal nerve divides into the internal and external branches. The internal branch further subdivides into two branches, one which innervates the epiglottis and the other which descends to anastomose with the posterior ramus of the recurrent laryngeal nerve. This branch of the superior laryngeal nerve also serves as a pharyngeal sensory nerve. The external branch supplies the cricothyroid muscle as the motor nerve. The recurrent laryngeal nerve divides into two branches at the level of the second tracheal ring. The first branch called the posterior ramus anastomoses with the superior laryngeal nerve as mentioned earlier. The second branch called the anterior ramus supplies the vocalis muscle [59]. All the intrinsic laryngeal muscles are innervated by the recurrent laryngeal nerve except the cricothyroid muscle which is innervated by the external branch of the superior laryngeal nerve.

2.1.4 Arterial blood supply

The larynx receives blood supply from the superior and inferior laryngeal arteries and to a small extent from the cricothyroid artery. The superior laryngeal artery is a branch of the superior thyroid artery which originates from the carotid artery. The inferior laryngeal artery is a branch of the inferior thyroid artery which originates from the subclavian artery. The cricothyroid artery branches from the superior thyroid artery. The three laryngeal arteries freely anastomose with each other.

2.1.5 Venous blood drainage

The superior laryngeal vein drains into the superior and middle thyroid veins which eventually empties into the jugular vein. The inferior laryngeal vein empties into the middle and inferior thyroid veins which eventually empties into the superior vena cava.

2.1.6 Lymphatic drainage

The larynx except the free margins of the vocal folds (which is our focus area for research) is richly supplied with lymphatic drainage. The area above the vocal folds drains into the superior and middle jugular nodes. The remaining part drains into the paratracheal lymph nodes, middle and inferior jugular nodes.

2.1.7 Physiology of phonation

Conceptual statement :- The biomechanics of the vocal fold tissue underlies the quality of voice.

The vocal folds are connective tissues located in the larynx which function to transduce aerodynamic energy from exhalation to acoustical energy for voice production. A healthy voice is contingent upon the balance maintained between the pressure exerted by the pulmonary system and the resistance offered by the vocal fold tissue [60]. In engineering terms, voice is a result of synchronous activity of the power source, oscillator and resonators [13]. The lungs, thoracic muscles, back muscles, diaphragm and abdominal muscles together constitute the power source which forces out the air during

exhalation. The larynx functions as the oscillator. The parts of the larynx above the glottis comprising pharynx, nasal cavity, oral cavity and paranasal sinuses together form the resonators. Phonation is the net result of the co-ordination of a multitude of factors like subglottic air pressure, glottal air flow, glottal resistance, vocalis muscle contraction, vocal fold tissue biomechanics, Bernoulli's effect, and acoustic coupling of the cavities above and below the glottis [61]. During quiet respiration the vocal folds are in an intermediate position with slight glottal widening during inspiration [59]. The vocal folds are medialized and elongated at the beginning of each phonatory cycle. The subglottic air pressure needed to initiate phonation is defined as the phonation threshold pressure [61, 62]. A rise in subglottic pressure just above the phonation threshold level which is approximately 7cm of water for conversational speech [13] pushes the vocal folds apart by progressively straining the vocal ligament (elastic component of the vocal folds) until a gap develops and the exhaled air begins to flow out thus vibrating the vocal mucosa (vibratory component of the vocal folds). The vibrating vocal mucosa gives rise to a mucosal wave (shear wave) which underlies the quality of voice. At this point the elastic properties of the vocal folds and the Bernoulli's forces generated by the air flow between the vocal folds begin to medialize the vocal ligament even as the vocal mucosa is still vibrating. One phonatory cycle is completed when the vocal mucosa snaps back to the midline eventually. Subglottic air pressure builds up again initiating yet another phonatory cycle. Movement of the lips and tongue change this sound to create individual speech sounds. The phonation threshold pressure is linearly related to the vocal fold tissue viscoelastic properties [7]. The fundamental frequency (pitch) of phonation

depends upon length and stiffness of the vocal fold tissue (mechanical properties), exhaled air pressure, and the mass of the vocal fold tissue [13]. Formant frequencies are achieved by altering vocal tract shapes with the help of extrinsic laryngeal muscles (formant frequencies are four or five resonant frequencies of the vocal tract [13]. The vocal fold tissue (*vocal mucosa*) vibrates at frequencies of 100 to 1000Hz at amplitudes of 1mm [11]. The *vocal ligament* experiences approximately 15% strain at normal phonatory frequencies [63].

2.2 ECM and biomechanics of phonation

Conceptual statement :- *The ECM composition of the vocal fold tissue is fundamental to the biomechanics of voice production.*

Briefly, the vocal fold tissue is a good example of a tissue whose form and function are inter-related. Most vocal pathologies arise from an altered ECM composition [2].

- In *engineering* terms, the viscoelastic properties of the vocal fold tissues are purely responsible for the normal vocal fold vibratory behavior.
- In *biological* terms, the unique matrix composition of the vocal fold tissue underlies the quality of human voice.
- In *layman* terms, the quality of human voice depends upon the vocal fold tissue architecture.

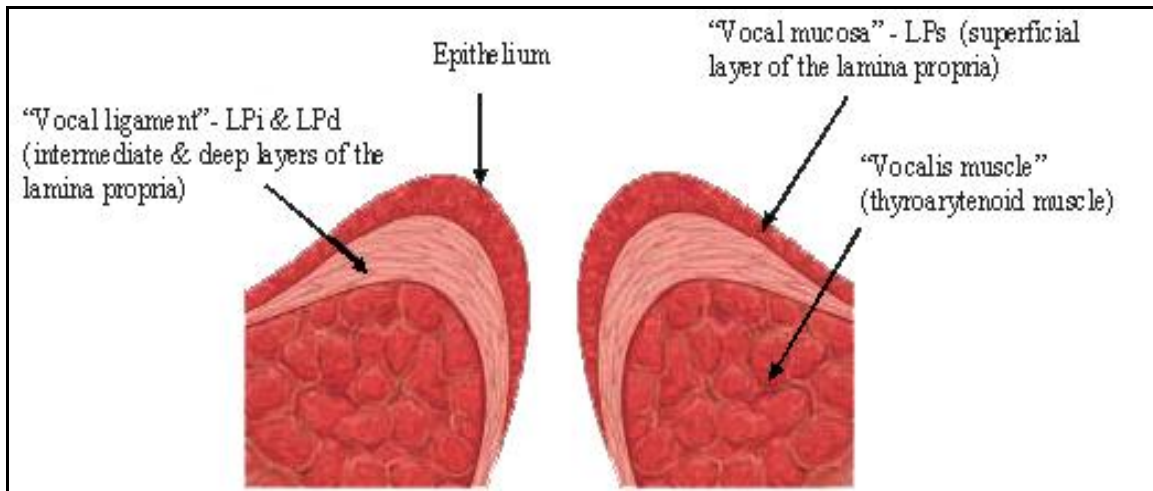


Figure 2.3 The multi-layered structure of the human vocal folds

The range of sounds a single pair of adult human vocal folds can produce is simply amazing especially in comparison to that of a musical instrument [64]. This ability or rather versatility stems from the fact that the matrix composition of the vocal fold tissue has tremendous functional significance in the context of phonation [65]. As indicated in figure 2.3, the adult human vocal fold is a highly differentiated membranous structure consisting of a lamina propria sandwiched between the stratified squamous epithelium and the vocalis muscle (thyroarytenoid muscle) [66]. Histologically the adult human vocal folds comprise six different layers :- epidermis (the outer epithelium), the basement membrane zone (BMZ), the superficial (LPs), intermediate (LPi), and deep (LPd) layers of the lamina propria and the vocalis muscle [2, 66-69]. The LPs is also known as the “Reinke’s space”. The biomechanical performance of the vocal fold tissue correlates with the anatomic arrangement of structural scaffolding proteins and this leads to a “cover-body” theory of vibration [68, 69]. The epidermis and LPs together constitute the

vocal fold “cover” which is popularly known as the vocal mucosa [69]. The LPi and LPd together constitute the vocal ligament. The vocalis muscle (thyroarytenoid muscle) forms the “body” of the cover-body structure. Animals and new born infants lack the vocal ligament [70-72].

2.2.1 Epidermis

The stratified squamous epithelium forms the epidermis or the outer cover of the vocal fold tissue whereas ciliated pseudocolumnar epithelium forms the outer covering of the remaining parts of the larynx [2]. This epidermis serves as the protective covering which gives shape and consistency to the underlying lamina propria. The epidermis is primarily cellular with the cells attached to each other through desmosomes (attachments between cytoskeleton of the adjacent cells). The ability to resist repetitive collisions the vocal folds are subjected to over several decades can be attributed to the presence of the desmosomes. The stratified squamous epithelial cells are metabolically active until desquamation unlike epithelial cells of the skin. Desquamated cells in the uppermost layer are replenished by the dividing basal cells. The luminal surface of these epithelial cells is covered with a microridge pattern. The microridged pattern may assist in increased mucus adherence, water absorption (due to an effective increase in surface area) and increased traction during vocal fold vibration. A mucociliary blanket (layer of mucus) which comprises the mucinous layer and the serous layer covers the epithelial lining [2, 73]. The mucinous layer which is the luminal layer prevents dehydration of the underlying serous layer and the epithelial cells. The serous layer which has a much

higher degree of hydration (in comparison to the mucinous layer) is in direct contact with the cilia which can beat freely within the less viscous serous layer than when it is in contact with the thick, viscous mucinous layer. This mucociliary blanket travels upwards in a circular fashion at the rate of 4 to 21 mm/minute in a normal person. One puff of cigarette, cold and dry conditions can slow down mucociliary clearance enough to expose the mucus to inhaled environmental toxins, inflammatory agents, allergens and dehydration [2, 74]. Thereby the mucociliary blanket is responsible for keeping the vocal fold healthy, moist and lubricated under normal conditions.

2.2.2 Basement membrane zone (BMZ)

It is also referred to as the submucosal BMZ. The BMZ is necessary for the ordered development of the multilayered vocal fold tissue [75]. The BMZ secures the overlying epidermis (stratified squamous epithelial cells) to the underlying lamina propria (primarily acellular amorphous ECM components) through anchoring fibers composed of collagen types IV and VII [2]. The BMZ is a four layered structure comprising the plasma membrane of the basal epithelial cells, the lamina lucida (immediately below the basal cells of the epidermis), the lamina densa and the sub lamina densa (above the LPs) [2, 75-78]. The anchoring filaments composed of collagen type IV anchor the hemidesmosome of the basal cells to the lamina lucida and the lamina densa [75]. The anchoring fibers composed of collagen type VII loop from the lamina densa through the sub lamina densa to the LPs and back again to the lamina densa. The concentration i.e. population density of anchoring fibers is genetically influenced [79, 80]. An average

person may have 80 to 120 anchoring fibers per unit area of BMZ whereas a person with a recessive gene for collagen VII may have only 40 to 50 anchoring fibers per unit area of BMZ. A person who is homozygous for the recessive gene will have almost no anchoring fibers [81]. Heparan sulfate proteoglycan is found in the BMZ, and it serves to bind collagen type IV, laminin, and fibronectin [4]. It is reported that in normal conditions the BMZ appears rectilinear but pathology lends a non uniform distorted appearance to the BMZ [75]. The extent of non uniformity explains the degree of structural tissue damage.

2.2.3 Lamina propria

In adults the lamina propria is a hypocellular structure whose ECM composition plays a significant role in maintaining normal phonatory voice. However, in newborns the lamina propria is characterized as a single layer of cells [82]. In adults the lamina propria is a loose connection of ECM components which comprises structural glycoproteins, fibrous proteins, non fibrous proteoglycans, glycosaminoglycans (GAGs) like HA, sparsely interspersed fibroblasts, myofibroblasts (differentiated fibroblast), and occasionally macrophages [2, 68, 69]. In 2001, Sato and co-workers, confirmed that some cells which had a stellate (star like) morphology with slender cytoplasmic processes (broadly classified as fibroblasts) were distinctly different from the conventional fibroblasts both in terms of morphology and differentiated functions i.e. protein expression [83-87]. These cells came to be known as vocal fold stellate cells named after their peculiar star-like morphology. The vocal fold stellate cells are widely

distributed in the macula flavae (junction/transition region between the vocal ligament and thyroid cartilage which bears maximum tension in the vocal ligament) but are absent in the LPs [83]. It is reported that the stellate cells actively synthesize elastic and collagen fibers and are responsible for the development of the vocal ligament after birth [71, 88, 89]. The interaction between structural glycoproteins like fibronectin and fibrous proteins like elastin and collagen serves to stabilize the matrix scaffolding of the biomechanically important lamina propria [2, 68, 83, 90]. Conventional fibroblasts are found in the vocal mucosa interspersed within the ECM. Originally, it was widely accepted that the bipolar spindle shaped fibroblasts produced the vocal fold tissue matrix [64, 65, 70, 91, 92]. However, in 1996 Pawlak and co-workers demonstrated cytoplasmic staining for proteoglycans in macrophages and myofibroblasts [4]. It was observed that the concentration of macrophages and myofibroblasts was highest in the superficial fifth and that of the conventional fibroblasts was maximum in the deepest fifth of the lamina propria [65]. Interestingly, women have a significantly higher concentration of macrophages in the superficial fifth of the lamina propria [65]. Macrophages are the cells which respond to and may cause inflammation. They are mainly found in the submucosal BMZ (between the epidermis and the LPs). The location of the macrophages suggests that they are present to counter agents like bacteria, viruses and other inhalants that cross the epithelium and can be harmful to the vocal fold tissue [2]. Macrophages produce and release the cytokine IL-1 which plays a central role in the mediation of inflammation. IL-1 causes the vocal fold fibroblasts to produce more HA and less versican [93, 94]. The myofibroblasts are a differentiated form of fibroblasts

containing smooth muscle alpha actin and are found in areas of damage and function as repair cells [95]. They are anchored to the matrix by a transmembrane complex between the intracellular and extracellular fibronectin fibers called a fibronexus [96]. The actin and fibronectin aid myofibroblasts in reorganization of the tissue during the reparative stage [97]. Interestingly, these cells are also found in normal human vocal folds. The concentration of myofibroblasts decreases as the depth of the vocal fold tissue increases (highest in the LPs and absent in the LPd) [2]. This indicates the presence of microtrauma and constant repair efforts in the vocal fold tissue as a result of the constant impact from phonation (normal use of vocal folds) [2, 65].

From the above observations one may infer that the synergistic action of the following helps in restoring the microscopic injury without any significant alteration in the vocal fold ECM composition :-

- myofibroblasts (elevate collagen levels leading to scar formation)
- increased synthesis of HA due to IL-1 activity (helps in retaining elevated levels of HA during the acute phase of wound healing to prevent scarring)
- deposition of fibronectin (mediates wound healing by acting as a chemoattractant for monocytes and fibroblasts, by promoting cell-matrix interactions through its cell and matrix macromolecule binding domains) [2, 90, 93, 94, 98, 99].

It is reported that microinjury repair can take up to 48 hours to heal [2]. However chronic vocal abuse may lead to the onset of vocal pathology due to a vigorous (macroscopic) unresolvable injury to the vocal fold tissue catalyzed by inadequate healing time.

One may thus infer that the macrophages, myofibroblasts, conventional fibroblasts and the vocal fold stellate cells together are actively involved in matrix synthesis of the vocal fold tissue.

2.2.3.1 Vocal mucosa

(a) ECM composition of the human vocal mucosa - The matrix of the vocal mucosa (epithelium and LPs) is abundant in glycoproteins like fibronectin, small interstitial proteoglycans like decorin, large proteoglycans like versican, GAGs like HA, and small amounts of elastic fibers like elastin and oxytalan [2-4, 6, 7, 90, 100]. Fibronectin is a ubiquitous glycoprotein which plays a vital role in cellular functions and presents binding sites for matrix macromolecules as well as cells [19, 90]. Fibroblasts deposit fibronectin in response to interstitial tissue damage [19]. Fibronectin binds to fibrous proteins like collagen and proteoglycans and thereby plays a vital role in vocal fold tissue matrix assembly. However, it was reported in 1999 by Chan and co-workers [101] that although abundant in the vocal fold tissue, fibronectin does not play a vital role in the maintenance of the vocal fold tissue viscoelasticity. It is counterintuitive that though fibronectin has binding sites for HA it does not affect the biomechanics of the tissue. The reason behind this may be the use of an inappropriate isoform, a smaller molecular weight or a lower approximation of the biological concentration of fibronectin [90]. On the other hand the sole function of fibronectin may be to assemble and maintain the matrix and the cells in the vocal fold tissue [90]. Decorin is found mainly in the LPs and its main function is to bind and regulate the thickness of collagen fibrils [2, 4, 7, 15]. Decorin mainly binds

collagen types I and II [4]. The presence of decorin in the LPs not only delays but also reduces the concentration of collagen fibrils in the vocal mucosa. HA differs from other GAGs in that it does not occur covalently bound to proteins, and is usually considered unique though it is described along with the proteoglycans in the context of the vocal folds [7]. HA is the only GAG present in the vocal fold tissue. HA is a porous, high molecular weight, polyanionic molecule which is responsible for regulation of osmosis by attracting sodium ions and therefore water. HA in turn controls the tissue viscosity by regulating the hydration levels of the vocal mucosa [2, 7]. Thereby HA lends the vocal fold tissue ability to resist the pounding and vibratory shocks sustained during phonation [7]. Thus, the viscoelastic properties of the mucosal wave (shear wave) which underlies the quality of voice is largely dependent upon the levels of HA in the vocal mucosa. HA is a space filling molecule which serves to regulate the fibrillogenesis of collagen in combination with decorin and thereby helps in regulating the size of the vocal mucosa [2, 4, 7]. In short, HA contributes to tissue viscosity and the initiation and propagation of vocal fold oscillations [102]. Versican appears to play a vital role in the lamina propria of the vocal fold. Versican possesses an HA binding domain and thus interacts with HA in the matrix of the vocal mucosa [4]. The HA levels in the vocal mucosa seem to correlate more with gender than with age. Males are reported to have three times more HA than females and subsequently have a thicker lamina propria [3]. This may contribute to increased molecular interactions leading to an increase in tissue elasticity and viscosity [103]. Elastic fibers like oxytalan and elastin are believed to be interruptions in elastogenesis varying in the degree of crosslinking [3, 104, 105].

Oxytalan, a fibrillar, hydrophilic protein contains very little amorphous elastic material and hence does not elongate under mechanical stress [105, 106]. Elaunin is an intermediary between fibrillar hydrophilic oxytalan and large amorphous hydrophobic elastic fibers. Elaunin fibers are shorter and smaller than complete elastic fibers [107]. Elaunin exhibits elastic properties intermediate to those of oxytalan and complete elastic fibers [105].

(b) Biomechanics of the human vocal mucosa - In terms of biomechanics, the vocal mucosa is isotropic due to the random arrangement of the matrix macromolecules [103]. The vocal mucosa (surface of the vocal fold tissue) propagates in the form of a shear wave (mucosal wave) during phonation. Steady shear deformation and oscillatory shear deformation are the two standard rheological techniques to assess the viscoelastic shear properties of materials. The human vocal mucosa is neither “Hookean elastic” (ideal solid) nor “Newtonian viscous” (ideal liquid) [103]. For a Newtonian fluid like water the steady-state viscosity is independent of the strain rate (it is dependent only on temperature). But non-Newtonian materials like biological tissues and polymeric materials have viscoelastic properties that depend upon the strain rate. The vocal mucosa is viscoelastic, exhibiting both elastic and viscous properties. Thereby, the viscoelastic properties of the human vocal mucosa are characterized rheologically using sinusoidal oscillatory shear deformation [103]. Rheological properties of the human vocal mucosa are a macroscopic manifestation of the microscopic and molecular arrangements. The viscoelastic shear properties of the human vocal mucosa are quantified in terms of complex shear modulus which includes the elastic shear modulus and the viscous shear

modulus. The elastic shear modulus is also known as the storage modulus and is a measure of the amount of energy stored in the vocal fold tissue during one cycle of oscillation/phonation. The viscous shear modulus is also known as the loss modulus which is a measure of the amount of energy dissipated in the form of heat during one cycle of oscillation/phonation. The viscoelastic shear properties of the vocal mucosa are often reported in terms of dynamic viscosity which is obtained by dividing the viscous shear modulus by angular frequency over the entire range of frequencies tested. The dynamic viscosity response of the human vocal mucosa shows a ‘shear thinning’ response (decrease in frequency is linear on a log scale) [103]. In some cases an order of magnitude difference has been reported between males and females [103]. This may be attributed to the gender related difference in ECM composition. The phonation threshold pressure and dynamic viscosity of the vocal mucosa share a linear relationship [7]. Vocal fold tissue viscosity and elasticity depend upon the ease with which slippage can occur between and within molecules. This in turn depends upon the intermolecular and intramolecular interactions between matrix macromolecules such as:-

- hydrogen bonds (electrostatic bonds)
- physicochemical interactions (covalent crosslinks)
- hydrophilic and hydrophobic interactions (interactions between polar and non polar functional groups)

Thereby, the concentration, molecular weight, and molecular structure of the matrix macromolecules which contribute to the 3D tissue architecture are the most important factors in regulating the viscoelastic properties of the vocal mucosa.

2.2.3.2 Vocal ligament

(a) **Matrix composition of the human vocal ligament** - The human vocal ligament comprises the LPi and LPd. It forms the transition region between the cover (vocal mucosa) and the body (vocalis muscle) of the vocal fold tissue. The vocal ligament runs between the anterior and posterior macula flavae [89]. The macula flavae is the junction/transition region between the vocal ligament and the thyroid cartilage, which bears the maximum tension in the vocal ligament. The anterior and posterior macula flavae are connected to the thyroid and arytenoid cartilages, respectively, and are 1.5mm x 1.5mm x 1.0mm in size. Conventional fibroblasts and vocal fold stellate cells make up the matrix producing cellular component of the macula flavae [89]. The cell density of the macula flavae is lesser than that of the LPs [64]. The fibroblasts and vocal fold stellate cells actively produce an ECM rich in elastic and collagen fibers. At the macula flavae ends of the vocal ligament, there are gradual changes in the stiffness of the junction region between the vocal ligament and the hard cartilages [108]. The macula flavae is present in new born infants but the vocal ligament is absent [70, 89]. The cell density in the macula flavae of infants is much higher than that of an adult [71]. It is widely accepted that phonation after birth stimulates the fibroblasts in the macula flavae which eventually leads to the development of the vocal ligament [89]. The human vocal ligament matures after puberty [109]. The macula flavae is essential for growth, development, and aging of the vocal fold tissue [71, 88, 89]. One might infer that the fibroblasts in the macula flavae produce collagen and elastic fibers in response to mechanical stress which is the epigenetic stimulus regulating the matrix composition of

the macula flavae itself and thereby the vocal ligament. The ECM of the vocal ligament is abundant in small interstitial proteoglycans like fibromodulin and fibrous proteins like elastin and collagen, small amounts of HA and glycoproteins like fibronectin [2-4, 6, 7, 14, 65, 90, 100]. Fibromodulin is concentrated in the vocal ligament and it serves to bind and regulate the thickness of collagen fibrils [2, 4, 7]. Interactions between glycoproteins like fibronectin and fibrous proteins like elastin and collagen provide scaffolding to the vocal fold tissue. The elastin and collagen fibers of the vocal ligament are also produced by the vocal fold stellate cells located in the macula flavae [83, 89]. The concentration of elastin fibers is reported to correlate more with age than with gender [110]. Native elastin which can otherwise be stretched to five times its original length without any loss of resilience can be stretched to only twice its normal length in the vocal fold tissue due to its association with collagen [92, 111, 112]. Collagen imparts tensile strength to the vocal ligament. Tensile strength arises from the crosslinking present within collagen which is due to covalent bonds formed between the lysine and hydroxylysine residues on adjacent molecules [111]. Lack of elastin and collagen may result in unco-ordinated vibration of the vocal fold tissue resulting in dysphonia [110]. Proteoglycans influence the interaction between elastin and collagen fibers [6]. Therefore, interactions between fibronectin, fibromodulin, HA, and primarily elastin and collagen impart elasticity and tension bearing properties to the vocal ligament.

(b) Biomechanics of the human vocal ligament - Young's modulus (elastic modulus) accurately represents the elasticity of the human vocal ligament [8]. Elasticity of the vocal ligament plays a key role in control of phonation since the mucosal wave first

begins from the deeper layers of the lamina propria, then propagates to (transverse direction), and along the surface (vertical direction). It was reported that the vocal fold tissue stress-strain relationship is linear at low strain levels (<15% strain) and non linear at higher strain levels [63]. Low strain levels (<15% strain) represent normal phonatory strain levels [8]. Min and co-workers, characterized the longitudinal elasticity of the human vocal ligament using excised tissue from human larynges and reported the Young's modulus to be approximately 33kPa at low strains (<15%) [63]. At higher strains (>15%) the Young's modulus values were 135kPa (25% strain), and 600kPa (40% strain). The elastic moduli of the human vocal ligament at higher strains (>15%) predominates over the vocal mucosal stress thereby playing a fundamental role in controlling frequency at high strain. Tran and co-workers, reported the transverse elasticity of the human vocal ligament using an in vivo measurement technique on human patients' vocal fold tissue under general anesthesia [8]. The recurrent laryngeal nerve was stimulated with 2 different currents 3.8mA and 3.2mA at 80Hz for a pulse duration of 1.5ms. At high stimulation the transverse elasticity was reported to be approximately 21kPa and at low stimulation it was reported to be 19kPa. Though there is a significant difference in the values of the longitudinal moduli and transverse moduli, one cannot infer that the vocal ligament is anisotropic (longitudinal vs transverse). Further studies need to be performed in order to characterize the transverse moduli at varying levels of stimulation. It is important to characterize transverse elastic moduli of the vocal ligament because initial mucosal wave movement (LPd to LPs) is in the transverse direction (though the surface mucosal wave propagates in the vertical direction).

From the ECM composition of the vocal mucosa and the vocal ligament it is evident that the vocal mucosa is sparsely populated with elastin and collagen fibers whereas the vocal ligament has an abundance of these fibers. The linear and non linear elasticity at low and high strains correlate to the unique collagen ultrastructure of the human vocal ligament [113]. The helical collagen fibers function as a buffer at low strains so that elongations of the vocal ligament occur without much resistance [113]. At high strains, the unique collagen structure absorbs the stress and serves as a shock absorber along the length of the fibers.

From these observations one might wonder what causes the matrix producing cells to produce a certain kind of matrix in the vocal mucosa (~1mm below the surface) and yet another different matrix in the vocal ligament (~3 to 4mm below the surface of the vocal folds). Chemical signals in different tissues are thought to determine the location and extent of ECM protein deposition [114]. However, bearing in mind the mechanical function and vibratory microenvironment of the vocal fold tissue, one might hypothesize that vibration and strain are the epigenetic stimuli regulating the unique matrix composition of the human vocal fold tissue (especially the lamina propria). In other words, differences in gene expression (ultimately leading to a correspondingly different protein expression assuming there are no factors causing post translational modifications) in the vocal mucosa and vocal ligament may be a direct result of differences in the magnitude of mechanical stimulation experienced by the vocal mucosa (vibration) and the vocal ligament (strain).

2.3 Aging and matrix regulation by fibroblasts

2.3.1 Why does our voice change (presbyphonia) as we age?

As we age, the membranous vocal folds shorten in males, the vocal mucosa thickens in females and edema develops in the LPs of both males and females [115]. It had been reported that non pathological loss of voice quality sets in every five years for males above fifty years of age [116]. Aging causes matrix producing fibroblasts to senesce thereby resulting in less efficient functions. In other words, as we age the density of cells in the vocal fold tissue and their cytoplasmic components like golgi apparatus and rough endoplasmic reticulum (site for active protein synthesis) decrease in number [88]. The number of fibrous proteins (elastin and collagen fibers) synthesized by the senescent resident cells of the macula flavae also decreases. This alteration in balance leads to a change in the biomechanical performance of the tissue, ultimately leading to an aging voice. The process of degrading old proteins and synthesis of new proteins is referred to as “ECM turnover”. ECM turnover slows down as age progresses. Fibroblasts are implicated in ECM turnover by virtue of the enzymes they secrete which in turn degrade the matrix proteins. These enzymes are called matrix metalloproteases (MMPs). MMPs include collagenases, gelatinases, and elastases. MMP-1 (collagenase) degrades collagen types I and III. MMP-2 (gelatinase) degrades collagen type IV and VII which are BMZ proteins. Elastases degrade elastin molecules. The catabolic activity of the MMPs is inhibited by the tissue inhibitors of metalloproteases (TIMPs) [117]. Vocal fold tissue ECM regulation involves the interplay between MMPs (degrade matrix proteins) and TIMPs (inhibit the activity of the MMPs) [2]. Unfortunately, studies examining the

interaction between the MMPs and TIMPs in the human vocal fold have not been performed [2]. However, such studies have been performed in animal models. In these models the production of collagen and MMPs decreases with age but the production of TIMPs remains relatively constant throughout the life span [2]. The cumulative physiologic effect of decreased protein and MMPs expression coupled with a constant TIMPs secretion is slower protein turnover. This causes the existing proteins to become older (more crosslinked in case of collagen and elastin) before they are replaced. A higher degree of crosslinking further delays degradation by normal processes due to increased intra and intermolecular bond formation and also hampers biological activity of these molecules [2]. Elastin molecules become less elastic and the collagen molecules become stiffer due to increased crosslinking [118]. Fibronectin concentration in the vocal fold tissue increases as a result of constant repair throughout a lifetime [90]. In the context of the vocal fold tissue, as one ages, slower matrix turnover coupled with elevated levels of fibronectin causes the vocal fold tissue to become less elastic and more stiff, signaling a change in the biomechanical properties of the tissue which ultimately leads to slight a change in voice or in more chronic cases leads to dysphonia. For example, a stiffer, thinner vocal fold tissue will cause the vocal folds to vibrate faster and with smaller amplitude.

2.3.2 Collagen and senescence

As mentioned earlier, collagen types I, II and III are found in the lamina propria and collagen types IV and VII are found in the BMZ. As we age, increased collagen crosslinking and reduced ECM turnover rate makes the lamina propria stiffer [6]. It was reported that infant vocal folds had about 51% of the collagen found in all adults and in geriatrics. There was no significant difference between adult and geriatric folds. However, female adult and geriatric vocal folds had approximately 59% of the collagen found in male adult and geriatric vocal folds i.e. male vocal ligaments showed about twice the concentration of collagen in the female vocal ligament [119]. Thereby, the concentration of collagen fibers correlates more with gender than with age.

2.3.3 Elastin and senescence

Hammond and co-workers, reported that infants (smaller, thinner elastic fibers) had approximately 23% and geriatrics (larger, thicker elastic fibers) had approximately 879% of the elastin found in a normal adult human vocal fold [110]. Consistent with this finding the geriatric vocal ligament was reported to be larger than that of infants and adults [110]. However, Sato and Hirano, reported that the concentration of elastic fibers in geriatrics is lesser than that of normal adults [120]. Hirano and co-workers, also reported a similar reduction in elastin levels when geriatrics were compared to adults [109]. These contradictory results have not been discussed by these groups in later publications. Gray and co-workers, hypothesized that variable observations could be

attributed to differences in the quantitation techniques, specimen number and racial variations among subjects [6].

2.3.4 Hormones and aging

Newman and co-workers, reported the presence of androgen, estrogen, and progesterone in the human vocal fold tissue in varying concentrations and locations thus lending a hormonal angle to the aging voice [121]. The concentration of the progesterone receptor was higher in younger subjects across both genders in comparison to older subjects. It was also reported that variations in the estrogen/androgen ratio was responsible for voice changes in post menopausal women. The concentration of androgen and progesterone receptors is higher in males than in females. It must be stated that the relation between hormone levels and aging is yet to be fully understood.

2.4 Vocal fold damaging factors

Vocal fold tissue damage can be brought about by a multitude of factors such as [2, 13-15, 65, 122, 123] :-

(a) Vocal misuse/overuse

- chronic vocal overuse
- excessive exposure to cold and dry environments
- smoking

(b) Non phonatory mechanical injury

- trauma (head or neck injuries)

- surgical intubation
- improper use of CO₂ laser during surgery

(c) Vocal pathology

- benign lesions (nodules, polyps and Reinke's edema)
- laryngeal cancer
- medical problems like allergies of the upper respiratory tract, nasal and sinus regions, and laryngopharyngeal reflux (gastroesophageal reflux)
- infectious laryngeal diseases, for example - respiratory papillomatosis

2.5 Diagnosis/Assessment of vocal fold tissue damage

(a) Vocal fold tissue examination - Assessment of voice problems includes a detailed evaluation of patient history, perceptual voice assessments, laryngeal endoscopy (rigid endoscopy or transnasal flexible endoscopy), examination of the vocal fold tissue vibratory pattern using a videolaryngostroboscope, evaluation of aerodynamic efficiency (information about the efficiency of the larynx), pulmonary function and acoustical voice analysis (information about the physical aspects of voice) [18, 60].

(b) Vibratory tests - Vibratory tests are conducted with the help of videolaryngostroboscopes, fiberoptic-laryngoscopy, electroglottography / laryngomyography or ultra-high speed photography [13, 18, 60]. This kind of physical examination facilitates detection of asynchronous vibrations, vibration asymmetries, structural abnormalities like small masses and submucosal scars. Transnasal flexible laryngoscopy

helps identify compensatory behavior of the extrinsic laryngeal muscles by detecting muscle tension dysphonia arising secondary to glottic incompetence [18].

(c) **Aerodynamic studies** - Aerodynamic efficiency of the vocal fold tissue is examined using a pneumotachograph which measures air volume, air flow rate and phonation time [60]. Respiratory function is analyzed using a plethysmograph. Increase in vocal fold tissue stiffness results in a decrease in both the mean air flow rate and air volume.

(d) **Voice analysis** - Minor changes in the vibratory pattern of the vocal fold tissue can be detected by careful voice/acoustic analysis [60]. Vocal pathologies tend to contract the vocal frequency range in an adult human patient. The patient's normal phonatory frequency (pitch) and vocal range can be assessed using frequency measurements.

2.6 Vocal pathologies

Rapid strides in communications during the 20th century and in the ongoing 21st century involving media like radio, television, telephone and computers which have brought about upliftment of our society are based on the impact of voice [60]. Urban pollution and high stress levels can cause irritation to the fine lining of the vocal fold tissues [60]. Rapid transportation resources result in lack of time for globe trotting performers (singers, actors and teachers) to acclimatize their voice producing organ.

The six most frequently reported dysphonia-inducing vocal pathologies are vocal fold scarring, nodules, polyps, Reinke's edema, laryngeal cancer, and vocal fold paralysis [124]. The three most commonly diagnosed vocal pathologies are benign lesions like vocal fold polyps, nodules and Reinke's edema [125].

2.6.1 Vocal fold scar

Scarring is implicated as the single largest cause for the loss of voice quality after surgery [18]. Scarring of the vocal fold tissue is a result of blunt trauma, penetrating neck injuries, iatrogenic injuries, surgical intubation, radiation therapy for removal of lesions, pro-inflammatory conditions like laryngopharyngeal reflux and laryngeal infections [13]. Vocal fold scar may or may not co-exist with benign lesions. The functional result of a vocal fold scar is dysphonia due to decreased ability of the stiff unresponsive vocal fold tissue to vibrate freely and rapidly during phonation coupled with complications like aspiration (related to glottic incompetence). When viewed by a stroboscope the mucosal wave produced by the scarred tissue appears to be irregular and reduced in both frequency and amplitude as compared to that of a normal vocal fold tissue. Scarring-induced reduction in vibrational amplitude of the mucosal wave leads to decreased intensity of voice. The phonation threshold pressure (subglottic pressure required to initiate phonation) is increased as a result of an altered ECM composition of the vocal fold tissue. Thereby, in addition to dysphonia, patients complain of vocal fatigue, breathlessness, and increased phonatory effort [18]. The subject will attempt to overcome scar induced voicing difficulties by :- (a) altering the length and tension (mechanical properties) of the vocal fold tissue with the help of the intrinsic laryngeal muscles and (b) by changing the position and shape of the larynx with the extrinsic laryngeal muscles to increase the phonation threshold pressure [13]. This leads to compensatory muscle tension induced dysphonia secondary to scar induced dysphonia at a later stage [18].

Scar tissue is seldom diagnosed in the nascent stage due to virtually no evidence of mass lesion or visible abnormality. Treatment of the scarred vocal fold tissue is one of the most challenging voice problems faced by otolaryngologists today [18]. Any kind of injury to the vocal fold lamina propria elicits a wound healing response which is characterized by disorganized deposition of collagen which eventually results in scar formation. The acute phase of wound healing which includes the proliferative phase is very important in the context of scar formation [48, 126]. The proliferative phase begins 2 to 3 days after initial injury and lasts for 2 to 3 weeks [48]. During the proliferative phase fibroblasts migrate into the site of injury and actively synthesize matrix comprising mainly collagen. HA being a space occupying molecule in the matrix, affects collagen fibrillogenesis [127]. The balance between matrix molecules (collagen vs HA) produced during the proliferative phase is the defining difference between scar formation and regenerative-type wound healing [128]. Adult dermal wound healing is characterized by scar formation attributed to decreased HA levels by day 10 [129]. Scarless fetal wound healing is attributed to consistent elevated levels of HA leading to regenerative repair [128, 130]. It has been reported that HA levels in the vocal fold wounds decrease to sub normal levels after day 5 of injury resulting in vocal fold scarring [131]. However, chronic vocal fold scars (2 to 6 months post injury) reveal HA levels similar to that of normal tissue [132-134]. As mentioned earlier, elevated levels of HA throughout the wound healing process would help in regulating the fibrillogenesis of collagen and could thus play a vital role in averting scar formation primarily due to its space occupying

property. In addition to being disorganized and scattered, elastin levels are decreased in matured scars (2 to 6 months post injury) [132, 133].

Physiological effects of scar formation :- Thus scar formation leads to an altered matrix composition of the vocal folds tissue which subsequently causes dysphonia.

2.6.2 Benign vocal fold lesions

Injuries like vocal fold polyps, nodules, and Reinke's edema (also known as vocal fold granulomas) arising out of chronic vocal overuse and inadequate hydration affect the lamina propria [14, 19, 65, 75, 135-137]. Vocal fold polyps and nodules are benign convex lesions along the midline of the vocal folds caused by increased mechanical stress generated by high velocity impact between the vocal folds during phonation [14]. Mechanical stress coupled with structural disruptions of the BMZ is the main cause of benign lesions which have a purely negative impact on the quality of voice. Among otolaryngologists there is no uniformity of vocabulary regarding benign vocal fold lesions like polyps, nodules, and Reinke's edema. This arises from the fact that clinical diagnosis is hampered by the varying macroscopic appearances of these non life threatening pathologies [125, 137]. Transformation of vocal polyps, nodules, and Reinke's edema from one form to another (for example from polyps to nodules) does not occur [137].

(a) Vocal fold polyps - Vocal polyps are defined as lesions on the anterior third of the vocal folds, often on the free edge, sessile or pedunculated, and very mobile when

pedunculated [136] (peduncle is a stalklike base to which a polyp or tumor is attached). The polyp-inducing phonotrauma is often an acute event which causes capillary damage leading to edema, bleeding, and fibrin leakage. Complete repair and recovery is often hampered by recurrent movements of the lesion during phonation, inducing recurrent capillary trauma. From these observations one might hypothesize that the age of the lesion cannot be estimated accurately since signs of recent/fresh bleeding could be misleading. Accumulation of HA [137] and fibronectin [135] around blood vessels is a unique feature of vocal fold polyps. A possible reason for elevated levels of HA around vasculature is the induction of fibroblasts and smooth muscle cells to deposit HA in response to inflammation and mechanical stress/trauma [138, 139]. The thickness of the submucosal BMZ remains unaltered [135]. Increased vasculature due to capillary proliferation lends a reddish color to the vocal polyps [136]. Severe disruptions of the endothelial lining are characteristic of vocal polyps. The combination of signs of recent/fresh bleeding, depositions of iron and fibrin, and thrombosis confirms the clinical diagnosis of vocal fold polyps [136].

(b) Vocal fold nodules - Vocal nodules are defined as small lesions occurring on both sides of the larynx, strictly symmetric on the border of the anterior and middle third of the vocal fold, and usually immobile during phonation [136]. These superficially located lesions move as fast as the frequency of phonation. Medially directed tearing forces generated as a sequelae of the Bernoulli's forces generated during the phonatory cycle are primarily responsible for vocal nodules. Glottic incompetence which preexists in this pathologic condition serves to increase the tearing forces/Bernoulli's forces [136]. Vocal

nodules cause submucosal BMZ thickening and less often capillary wall thickening [56]. Nodules are characterized by increased amounts of collagen type IV and fibronectin in the BMZ and the LPs [2, 135]. This kind of protein deposition is indicative of a repetitive injury to the tissue which results in abnormal and incomplete healing eventually leading to scar formation [2]. Elevated fibronectin levels in vocal nodules cause a reduction in mucosal wave motion [90]. Vocal nodules is a pathology that is more common in women than in men [3]. The combination of BMZ thickening, absence of hemorrhage, and absence of edematous lakes (increased levels of vasculature) confirms the clinical diagnosis of vocal fold nodules [136]. Women have a higher fundamental frequency of vibration than men. The higher frequency leads to shocks of higher impact when the vocal folds collide during the phonatory cycle. Also, three times higher HA content in the vocal fold tissue plays a vital role in preventing vocal nodules in men [3]. Increased capillary proliferation is not observed in vocal nodules [136]. Increased capillary thickness and disorganized fibronectin deposition is characteristic of vocal nodules [135]. No endothelial lining disruptions were observed in vocal nodules [136]. It is hypothesized that the perpendicular orientation of the elastic fibers in these lesions may be induced by the tearing/Bernoulli's forces [137].

(c) **Reinke's edema** - Reinke's edema is defined as a unilateral or bilateral swelling of the vocal fold, filled with fluid, sessile, and very mobile during phonation [136]. Reinke's edema swellings are reported to be white in color. During phonation the motion of this lesion is far more coordinated than that of a polypoid lesion. The edematous outgrowth is observed to roll over the medial edge of the vocal fold once every phonatory

cycle. Talkative people and heavy smokers are almost exclusively vulnerable to Reinke's edema though smoking has not been implicated as the leading trauma to this pathology [137]. This pathology is not gender specific. Reinke's edema is characterized by an increase in the thickness of the vocal mucosa, a decrease in tissue viscosity, an increase in fibrin and fibronectin deposition [7, 75, 90, 135-137]. Reinke's edema is associated with an increased mucosal wave pliability which can be attributed to elevated levels of fibronectin. It may seem counterintuitive that elevated levels of fibronectin in two different pathologies are responsible for two contrasting outcomes. Fibronectin isoforms could be a possible explanation for this observation [90]. Increased capillary proliferation is not observed in Reinke's edema [136]. But the vessel walls show an increase in thickness i.e thickness of the vessel wall is greater than the normal capillary basement membrane. Pericyte recruitment is also observed [136] (Pericytes are slender, contractile, mesenchymal-like cells found in close association with capillary walls. They are relatively undifferentiated and may differentiate into fibroblasts, macrophages, or smooth muscle cells (from Stedman's Medical Dictionary, 26th edition)). Slight disruptions of the endothelial cell lining are observed but these disruptions are much smaller than the those observed in case of polyps [136]. Smoke induced trauma is not severe enough to induce endothelial lining damage. The combination of submucosal BMZ thickening, edematous lakes, erythrocyte extravasation, and increased thickness of submucosal vessel walls confirms the clinical diagnosis of Reinke's edema.

Histological evaluations of vocal fold tissues afflicted by polyps, nodules, and Reinke's edema reveal increased or decreased levels of fibronectin. Increased fibronectin

deposition is a precursor for increased collagen deposition which heralds the onset of scar formation [19]. Interestingly, benign vocal lesions like polyps, nodules and Reinke's edema may or may not exhibit scar formation [19]. Abnormal levels of fibronectin in these pathologies indicate that a sufficient extent of matrix disruption had been caused and that the cellular response to bring about matrix repair was initiated. This fibroblastic response (increased fibronectin deposition) may be downregulated before scar formation sets in. But it must be noted that these benign neoplastic lesions can co-exist with vocal fold tissue scarring.

(d) Vocal fold cyst - A vocal fold cyst is defined as a unilateral undulation with a smooth surface, immobile during phonation, usually on the middle third of the vocal fold [136]. It is characterized by a normal or slightly thickened submucosal BMZ and a fairly normal distribution of fibronectin [135].

(e) Vocal fold pseudocyst - A vocal fold pseudocyst is defined as a swelling resembling a cyst, usually located on the junction of the anterior and the middle third of the vocal fold, the macroscopic wall of which is thin and translucent [136].

These observations suggest that the initiating trauma for vocal polyps is acute phonotrauma, cigarette smoke for Reinke's edema and tearing forces (Bernoulli's forces) for vocal nodules [136]. Vocal polyps are mechanically the most compromised of the three common vocal pathologies. Continual forces maintaining these lesions could cause thickening of the capillary basement membrane and persistent endothelial damage may lead to platelet adhesion and thrombosis. In summary, these vocal pathologies are the human vocal fold tissue (primarily the lamina propria and BMZ) response to

phonotrauma. Another striking observation is that the degree of BMZ disruptions witnessed in vocal pathologies is more severe than that witnessed in pathologies of any other organ. This reveals the extent of the destructive nature of repetitive mechanical stresses the vocal fold tissues are subjected to during a lifetime of phonation which hampers the wound healing process.

Finally, phonotrauma to the vocal fold tissues induces BMZ disruption and injury to the vocal mucosa. Vocal pathologies arising out of incomplete wound healing lead to dysphonia secondary to altered mucosal wave pliability or decreased vocal fold tissue vibration brought about by either scar formation (disorganized collagen deposition) or excessive fibronectin deposition (biological characteristics of strength, binding and adhesion do not contribute to efficient tissue pliability [19]). Benign vocal fold lesions may or may not co-exist with scarred vocal fold tissue.

Physiological effects of benign vocal fold lesions:- Polyps, nodules, and Reinke's edema lead to dysphonia secondary to an altered ECM composition of the vocal fold tissue.

2.6.3 Malignant vocal fold lesions and infectious diseases

Malignant tumors due to neoplastic lesions are detected by biopsy, direct laryngoscopy, and physical examinations. Early and advanced neoplastic lesions may partially or completely prevent (metastasis into vocalis muscle) vocal fold motion. Treatments include surgical removal of lesions and non surgical options such as radiation and chemotherapy. Surgical removal of malignant lesions may be followed by dysphonia

secondary to scar formation. Radiation therapy induced swelling and dryness results in destruction of the mucosal lining thereby exposing the vocal fold tissue to toxic inhalants and inflammatory agents which eventually damage the tissue. Respiratory papillomatosis is an infectious disease characterized by wart-like growths (papillomas) on the surface lining of the respiratory tract, which includes the throat, larynx, and trachea. The human papilloma viruses also known as genital warts cause respiratory papillomas. These papillomas may be mobile and pedunculated (slim stalk) or sessile (stalkless). Treatment methods include surgical removal of the papillomas and therapies to slow or stop viral regrowth. Till date none of the techniques have been able to prevent recurrence of respiratory papillomatosis. Surgical removal of the papillomas may cause dysphonia secondary to scar formation due to lack of regenerative-type wound healing.

2.6.4 Sulcus vocalis

Sulcus vocalis, a genetic disease/condition reveals bowed vocal folds [140]. This pathology presents a total loss of the LPs or a depression or vergeture extending into the vocal ligament or sometimes even into the vocalis muscle. Also in some cases of sulcus vocalis, the epithelium is directly attached to the vocal ligament [141]. Sulcus vocalis pathology presents increased BMZ thickness and a lack of normal elastic and collagen fibers in the lamina propria [140]. Sulcus vocalis is classified into three types of which types II and III can be treated clinically and are characterized by replacement of normal lamina propria by scar tissue [142]. Clinically, sulcus vocalis presents bowed shaped

vocal folds leading to glottic incompetence, breathy voice, decrease in maximum phonation time, and vocal fatigue.

2.6.5 Vocal fold paralysis

Branches of the vagus nerve which innervate the intrinsic laryngeal muscles are susceptible to injury due to trauma, surgery, or other causes. Unilateral vocal fold atrophy occurring secondary to recurrent laryngeal nerve paralysis results in highly attenuated motion of the mucosal wave. Partial existence of the mucosal wave could be attributed to the cricothyroid muscle which serves to adjust the tension of the vocalis muscle (thyroarytenoid muscle) [143]. Vocal fold paralysis also results in bowed vocal folds due to symmetric muscular atrophy eventually causing dysphonia (absent mucosal wave) and aspiration due to glottic incompetence. It is thereby evident that vocal pathologies arising out of muscular dysfunction/dystrophy mostly have a neuromuscular basis. Such disease conditions are beyond the scope of this review since our research objective does not focus on pathologies of neurologic origin.

2.7 Current treatment techniques

A normal phonatory voice is contingent upon the existence of near perfect approximation of the following four vocal fold tissue properties such as:- (i) mass, (ii) elasticity (stiffness), (iii) resistance, and (iv) symmetry. A variety of techniques have been developed for restoration, preservation, and improvement of laryngeal and vocal fold tissue function. Currently, voice/speech therapy, laryngeal reinnervation, and vocal

fold medialization/augmentation (injections) procedures are used to treat vocal pathologies. The various surgical procedures for voice improvements are collectively termed as “phonosurgery”. Phonosurgical techniques include implantation of prosthetics, plastics, and reconstructive laryngoplasty using both synthetic and natural materials.

2.7.1 Voice therapy

Voice therapy combined with the use of antibiotics and steroids is the initial line of treatment for vocal fold scars [13]. Mild scar-related loss of frequency, and voice quality can sometimes be ameliorated with voice therapy. Under conditions of significant scarring (scars take approximately six months to mature into a stiff white mass) voice therapy may not be very effective. Voice therapy is effective in treating dysphonias secondary to compensatory behavior of the extrinsic laryngeal muscles [18]. Voice therapy is ineffective when presented with pathologies like vocal nodules, polyps and Reinke’s edema despite rigorous adherence to the prescribed habits [19]. Surgical procedures immediately followed by a voice therapy program can effectively help in improving and restoring normal vocal behavior [13]. The voice therapy program takes into account scar-induced factors like vocal fold incompetence due to increased stiffness of vocal mucosa, reduction of mechanical decoupling of the vocal mucosa and vocalis muscle, and turbulence in the exhaled air stream. Singing voice therapy in combination with conventional voice therapy is a new method of voice rehabilitation [18]. This method involves warm-up exercises traditionally used by vocalists to enhance voice efficiency.

2.7.2 Laryngeal reinnervation

Injuries to the superior or the recurrent laryngeal nerves may result in compromised glottic function. The superior and/or recurrent laryngeal nerves can be injured during surgical procedures or may be subjected to traumatic crush or stretch injuries [144]. This technique involves use of a highly modified diagnostic microscope to detect small lesions in the vocal fold tissue [60]. Laryngeal reinnervation involves a careful microscope-aided search for the two severed ends of the nerve and precise approximation of the freshened nerve endings [60]. Currently, various research groups are focused on implantation of nerves into the different muscles and the identification of specific nerve bundles in the vagus nerve for reconstructive surgery. Reinnervation of the paralyzed vocal fold tissue is achieved by anastomosing the recurrent laryngeal nerve to the ansa hypoglossi (bunch of nerves which innervate the extrinsic laryngeal muscles) [145]. This method is reported to yield good voice quality since it restores tension to the vocal ligament and maintains bulk of the vocalis muscle. This method facilitates adduction of the vocal folds by pulling the back ends of the arytenoid cartilages apart. Thereby motion of the paralyzed vocal folds may be restored. One disadvantage associated with laryngeal reinnervation is that the ends of the nerves to be anastomosed may not be readily available, for example the posterior stump of the recurrent laryngeal nerve may be deeply embedded in a scar tissue following tissue response to the inducing trauma. Laryngeal reinnervation procedures do not result in immediate improvement of glottic functions [143]. Therefore resorbable gelfoam paste is injected into the paralyzed

vocal fold tissue to provide temporary medialization of the vocal fold tissue. Benefits of laryngeal reinnervation procedures usually manifest themselves within 2 to 4 months.

2.7.3 Vocal fold medialization/augmentation procedures

Vocal fold augmentation procedures involve medialization surgeries and augmentation injections. Payr in 1915, for the first time brought about vocal fold medialization by a technique called laryngeal framework surgery using a medially depressed pedicled cartilage flap [146]. Thereafter, Isshiki reintroduced, and popularized laryngeal framework surgical techniques (Thyroplasty type I) in 1974 [147], and also refined it in 1989 [148]. Koufman refined the technique involved in laryngeal framework surgery and popularized it in the USA [149]. Thyroplasty type I necessitates external or internal incisions and the procedure is carried out under general anesthesia. Thyroplasty type I involves creation of an incision in the thyroid cartilage which facilitates insertion of a space filling material (usually an inert substance) to push the atrophied vocalis muscle (thyroarytenoid muscle) thereby medializing the vocal folds. In other words, this technique is used for ameliorating glottic incompetence arising out of pathologies such as unilateral paralysis and vocalis muscle dystrophy using silastic and autologous cartilage [147]. Thyroplasty type I is effective in realizing vocal fold medialization and thus restoring laryngeal function when injection augmentation fails to bring about a reparative effect. The positive effects of this kind of medialization surgery are limited since the normal viscoelastic properties of the vocal mucosa are not restored [150]. Vocal fold medialization surgeries aim to restore original vocal fold tissue geometry in an attempt to

cure voice impairment. Medialization procedures entail the use of a non physiological, alloplastic implant which may result in problems like obstruction of airway due to migration or extrusion of the implant [151]. Vocal fold medialization procedures are recommended for patients presenting glottic incompetence of 1.5mm or more [13]. It must be noted that these medialization procedures are aimed at vocal improvement by ameliorating glottal incompetence and are not aimed at alleviating pathology [18]. It is widely accepted that only post voice therapy must scar tissue be subjected to medialization (augmentation) procedures. Medialization procedures achieve a reduction in phonatory effort associated with glottic incompetence but have no improvement in voice quality. For glottal gaps measuring lesser than 1.5mm augmentation injections are recommended [13]. Medialization procedures serve to increase the mass of the vocal fold tissue. Medialization procedures must be followed by voice and singing therapy during the immediate post-operative period [18]. Medialization surgeries and injection augmentation procedures have employed a wide variety of natural and synthetic materials to achieve glottic closure and restoration of the mucosal wave. Vocal fold medialization procedures began in 1911 when a German scientist, Bruning, injected paraffin into the vocal fold tissue seeking a cure for voice impairment [152]. The medialization materials are assessed in terms of Arnold's criterion which states that an ideal vocal fold augmentation material should be easily injectable, long lasting and well tolerated by the host tissues [153]. Material resorption and need for re-injection were the disadvantages associated with the use of natural materials like collagen, fat, fascia, and HA [41]. The

synthetic and natural materials used for vocal fold medialization will be discussed in the following section.

2.7.3.1 Synthetic materials and metals used for vocal fold medialization purposes

Injection laryngoplasty to correct glottic incompetence began in 1910 – 11 when a German otolaryngologist, Wilhelm Brunings injected paraffin into the vocal folds [144]. Since then paraffin injections have been discarded due to problems associated with implant extrusion and paraffinoma formation [154, 155]. The injected paraffin also caused problems by migrating to other tissues of the head and neck via the lymph nodes. This led to the discontinuation of vocal fold injections. The criterion for an ideal vocal fold implant as described by Godfrey Arnold is that the injected material should be injectable, long lasting and well tolerated by the host tissues. In 1955, Godfrey Arnold revived the dormant technique of vocal fold tissue rehabilitation by injecting autologous cartilage paste into the vocal fold tissue using the Brunings injector. However, problems like donor site morbidity and tedious purification processes led to the need for synthetic materials. Teflon was the first synthetic material to be injected into the vocal fold tissue. Teflon paste injections for vocal fold applications were introduced by Arnold in 1962. Teflon did not satisfy the criterion for an ideal vocal fold implant. Thereafter, collagen, fat, fascia, silicone, and HA have been used for vocal fold injection augmentation. In 1985, Ward and co-workers [144], developed a new technique for injecting augmentative (space fillers) materials into the vocal fold tissue. This technique which involves introduction of the material into the vocal fold tissue via transcutaneous placement of a

needle through the cricothyroid membrane allows vocal fold injection in almost any patient. A wide variety of materials like ceramics, hydroxyapatite, Teflon, ePTFE, PET, silicon, titanium, and vitalium have been used for vocal fold medialization procedures.

(a) Teflon (polytetrafluoroethylene - PTFE) - Teflon paste was first injected into the vocal folds by Arnold and co-workers., 1962, in an attempt to bring about vocal fold medialization [20, 21]. Since then Teflon (Teflon®, Mentor, Santa Barbara, CA) has been used to cure glottic incompetence and restore mucosal wave motion in paralyzed vocal fold tissues [41]. If the amount and technique of Teflon injected are perfect then excellent phonatory results follow [143]. Under-injection results in a hoarse breathy voice while over-injection may cause breathlessness and problems associated with reduction in glottic aperture. Excessively injected Teflon may be removed by a spot curing CO₂ laser or tracheotomy which may eventually lead to dysphonia secondary to scar formation. Extrusion from the implant site can cause obstruction of the airway [22, 156-159]. Migration from the implant site into regional lymph nodes resulted in granuloma formations. Teflon injections result in foreign body reactions (due to phagocytosis of Teflon particles by macrophages) and tissue fibrosis [160, 161]. Once injected, removal from the injected site is very difficult due to significant soft tissue reaction. Teflon injections were dispensed by a Bruning's injector and were targeted at the vocalis muscle (thyroarytenoid muscle) [162]. The main disadvantage of Teflon and most other non physiological materials is that they can never restore the normal mass and tension (mechanical properties) of the native vocal fold.

(b) ePTFE (expanded PTFE) - ePTFE (Gore-Tex®, WL Gore, Flagstaff, Az) is implanted into the vocalis muscle (thyroarytenoid muscle) and not injected like Teflon. Thus ePTFE implantation is classified as a laryngeal framework surgery or more popularly thyroplasty type I. ePTFE was widely used for cosmetic purposes but in 1997-98 its application was extended to curing vocal fold insufficiencies by McCulloch and co-workers [25]. This group implanted a 25mm long, 2mm wide, and 0.6mm thick strip of ePTFE, folded over 5 times into the vocalis muscle (thyroarytenoid muscle). Histological analysis of the explanted graft revealed the presence of isolated foreign body giant cells and a thin fibrous capsule around the ePTFE implant. It was reported that after 6 months when the rabbits were sacrificed, the implants were removed fairly easily but with application of slight traction. From these observations one may infer that the slight amount of tissue ingrowth (due to the porous nature of ePTFE) prevents implant extrusion. It was observed that unlike Teflon, ePTFE is less susceptible to granuloma formation. In 2000, Tamplenizza and co-workers [163], injected rolled ePTFE tubes (2.5mm average diameter) through a 4mm fenestration in the thyroid cartilage into the vocalis muscle (thyroarytenoid muscle). The tubular shape combined with the porous nature of the implant facilitated extensive tissue ingrowth which in turn anchored the it to the implantation site and prevented problems like airway obstruction associated with migration and extrusion [164]. Thus implanted ePTFE was reported to provide appreciable phonatory improvement over traditional thyroplasty (using Teflon) [165]. The ePTFE implant is easy to handle, and unlike other materials its shape can be altered even after placement in the implant site [24]. Implantation of ePTFE can be easily

combined with another procedure like arytenoid adduction to restore glottic closure and its easy removal from the site of implantation is a real advantage. Zeitels and co-workers [166], and Giovanni and co-workers [124], have reported vocal fold medialization procedures with minimal complications [24]. None of the above studies have reported problems associated with implant extrusion and migration.

(c) Calcium hydroxyapatite - Calcium hydroxyapatite is a biologically inert ceramic composed of phosphorous and calcium [167]. Calcium hydroxyapatite gel (Radiance®, BioForm, Frankesville, WI) is FDA approved for vocal fold augmentation applications [168]. This preparation comprises calcium hydroxyapatite spherules dispersed in a mixture of water and glycerin and is easily injectable and non resorbable [41]. Calcium hydroxyapatite spherules of varying sizes are available :- (a) Standard radiance spherules measure between 75 and 125µm and can be injected through a 21 gauge needle, (b) Radiance FN measures between 25 and 45µm and can be injected through a 27 gauge needle. These particle sizes prevent phagocytosis of the implant material. One of the main indications for calcium hydroxyapatite based injections is unilateral paralysis and presbyphonia due to bowed vocal folds [168]. Calcium hydroxyapatite gel is injected deep into the lateral portion of the vocalis muscle (thyroarytenoid muscle). Calcium hydroxyapatite is reported to be non inflammatory and non antigenic since it is formed from calcium and phosphate ions which exist normally in our body [167]. Chhetri and co-workers, performed a time course study evaluating the calcium hydroxyapatite gel (Coaptite®, BioForm, Frankesville, WI) implant performance in canine vocal folds upto a period of 12 months [26]. The spherules were 38 to 63µm in size dispersed in a

hydrogel (70% volume) comprising water, glycerin, and 3% carboxymethylcellulose which could be injected with a 23 gauge needle. Histological evaluation revealed some foreign body giant cells but no other signs of chronic inflammation. Post implantation, a decrease in the degree of medialization/increase in glottic incompetence was observed progressively over the various time points (1, 2, 3, 6, 9, and 12 months). This is due to resorption of the aqueous based carrier vehicle leading to increasing compactness of the implant material.

Thereby, one may infer that since over-injection may cause airway obstruction and associated problems, re-injection may be the only way to circumvent increasing glottic incompetence. But unlike injecting natural materials which are resorbed by the body calcium hydroxyapatite can be injected a certain limited number of times since it is not resorbed by the body. Contrasting opinions exist about the need for over-injection of calcium hydroxyapatite into the lateral aspect of the vocalis muscle (thyroarytenoid muscle). One might infer that experience is the best teacher with calcium hydroxyapatite injections for vocal fold medialization procedures.

Rosen and Thekdi, used both the endoscopic vocal fold injection (EVFI) and percutaneous vocal fold injection technique (PVFI) in an attempt to compare the efficacy of both the techniques for calcium hydroxyapatite injections into the vocalis muscle (thyroarytenoid muscle) or the thyroid cartilage [167]. They reported that the EVFI provided more precise placement of the implant material.

(d) Polydimethylsiloxane (PDMS) – PDMS was first used for vocal fold augmentation laryngoplasty in 1983 [169]. PDMS gel (Bioplastique ®, Bioplasty, Geleen ,

Netherlands) is both readily injectable and non resorbable. Unlike Teflon, the PDMS particles do not cause granuloma formation since they are too large to be taken up by macrophages [41]. PDMS gels alleviate glottic incompetence but the improvement in physical aspects of voice is not significant due to reduced mucosal wave pliability. This occurs due to the lack of approximation of the implant viscoelastic properties to that of the human vocal mucosa. Therefore, PDMS injections were limited to the LPd. PDMS based implants elicit minimal to no soft/host tissue response and can be removed fairly easily from the implant site. PDMS based materials are widely used in Europe but do not have FDA approval for use in the USA [41].

(e) **Silicon** - Silicon is one of the most frequently employed materials for vocal fold medialization [149]. Silicon poses problems with positioning and placing the implant in the site of interest [27]. If the implant dimensions do not match those of the implantation site then a new implant has to be prepared. Problems associated with migration of silicon from the implant site have also been reported [13]. Carcinogenic properties lend yet another disadvantage to silicon based vocal fold medialization.

(f) **Titanium** - In 1999, for the first time titanium was implanted into the vocalis muscle (thyroarytenoid muscle) and anchored to the thyroid cartilage (to prevent implant migration) by Friedrich and co-workers [170]. The rationale behind using titanium for medialization of the vocal fold tissue was retention of the lamina propria tissue architecture. Unilateral vocal fold paralysis and bowed vocal folds were indications for titanium based medialization of vocal folds. Titanium implants may be used in combination with another technique like arytenoid adduction to bring about glottic

closure and thereby improvements in voice. Schneider and co-workers, reported observations from a study involving titanium implants in human subjects suffering from incomplete glottic closure secondary to unilateral vocal fold paralysis [171]. The length of the implant was 15mm for men and 13mm for women. They observed an improvement in the motion of the mucosal wave of all patients 1 week after the procedure. The fundamental frequency of normal phonation and the voice intensity were unaffected by the medialization procedure.

(g) **Dacron (polyethylene terephthalate - PET)** – Keskin and co-workers reported observations from a study involving injection of PET into the vocal fold tissue of a rabbit model [27]. Histological evaluation on excised tissue 6 months after implantation revealed a discontinuous fibrous capsule surrounding the implant. Macrophages invaded the network structure of PET through the areas where capsule continuity was compromised which eventually resulted in foreign body giant cells surrounding PET fibrils. This study concluded that PET is inferior to titanium in terms of vocal fold tissue implant applications.

(h) **Gelfoam** - Gelfoam powder (prepared from gelatin) is mixed with saline and injected into the vocal fold tissue for temporary medialization [172]. Gelfoam (UpJohn, Kalamazoo, MI) has been used to cure glottic incompetence in vocal fold tissues afflicted by paralysis. However, it is reported to last only for approximately 2 months before it is resorbed (6 to 8 weeks). This entails over-correction or even multiple re-injections.

(i) **Lactosorb (Polylactic acid (PLA)/Polyglycolic acid (PGA) copolymer)** - Lactosorb is a PLA/PGA copolymer [173]. PLA (MW 180 – 530kDa) is a pale, semicrystalline

material whereas PGA (MW 20 – 145kDa) is a hard, crystalline polymer which is insoluble in most solvents. A PLA/PGA copolymer has variable degradation rates depending upon the ratio of PLA to PGA. Copolymer degradation occurs via cleavage (hydrolysis) of the ester bonds. Additionally, molecular weights of the participating polymers, crystallinity, and degree of purity of the material contribute to the degradation rates. PLA has a longer half-life and lesser strength in comparison to PGA which has shorter half-life and more strength. Lactosorb comprises 20% PGA and the remaining 80% is PLA, thereby it retains 80% of its strength after 6 weeks. Dufresne and Lafreniere, implanted Lactosorb into the vocalis muscle of rabbits and evaluated the implant morphology (implant resorption), host tissue response, maintenance of vocal fold medialization, and airway patency after 1, 3, 6 and 9 months [173]. Briefly, the recurrent laryngeal nerve was transected, a Lactosorb implant of dimensions 5mm x 4 mm x 3mm was introduced into the vocalis muscle (thyroarytenoid muscle) and secured to the thyroid cartilage using nylon sutures. Gross examination revealed a thick fibrous capsule surrounding the implant post 1 and 3 months. A foreign body response characterized by the presence of small number of macrophages and multinucleated giant cells was observed in addition to cells like lymphocytes, and fibroblasts. One might infer that these macrophages and giant cells were functioning to resorb the implant. Yet there was no significant change in the implant bulk volume and vocal fold medialization was maintained. Replacement of muscle tissue by adipose tissue, and a decrease in muscle fiber size suggested vocalis muscle atrophy. Post 6 months of implantation, a thin fibrous capsule surrounded the barely visible implant. A large number of macrophages and

foreign body giant cells were once again identified at the host-implant interface suggesting further implant degradation was in progress. Post 9 months of implantation the implant was completely resorbed (macroscopically and histologically invisible) yet the augmented vocal fold maintained both its bulk volume and airway patency. The authors hypothesized that the observed adipose cell invasion, muscle cell swelling, and thyroarytenoid hypertrophy may have contributed to sustained vocal fold medialization despite complete resorption of the implant.

This material does possess some disadvantages in terms of processability. Lactoborb is very hard and brittle which makes shaping at the time of implantation impossible, for example heating may cause the material to melt away. Lactosorb will need to be manufactured according to specific dimensions dictated by patient needs and the slightest need for alteration entails preparation of a new implant.

The above study reports the presence of huge amounts of uncharacterized intercellular material within the implanted muscle tissue. One might hypothesize that this is an inflammatory response to the acidic degradation products produced by the copolymer degradation. Since the study spanned nine months it is not possible to comment on the long-term fate and efficiency of vocal fold tissue medialization. Also, implantation in a canine model will throw some light on the effect of Lactosorb implantation on the physical aspects of voice. Considering a hypothetical situation wherein Lactosorb is implanted into the vocalis muscle of a patient with severe gastroesophageal reflux. Acidic conditions (pH of gastroesophageal reflux is between 3 and 6) could bring about an autocatalytic effect i.e. degrade the implant faster than

normal. Degradation of the implant material could result in a heightened local inflammatory response which may eventually lead to scar formation. This may cause dysphonia secondary to scar and glottic incompetence.

(j) **Botulinum toxin** - Benign lesions like polyps, nodules, and Reinke's edema as mentioned earlier are the result of increased mechanical stress due to the 'hammer-on-anvil' pounding effect on the vocal fold tissue during phonation. Otolaryngologists seek to alleviate vocal impairments arising from these lesions through surgical or CO₂ laser - based excision of these convex lesions. But it must be stated that surgical or cold excisions of these lesions aim to cure the symptom and not the cause. In other words, excision based methods eliminate the lesion but leave the cause unaltered. In the case of these vocal fold granulomas the main cause is chronic vocal abuse. Even complete vocal rest does not prevent vocal fold adduction and arytenoid contact which occurs during coughing and swallowing. In an attempt to completely avoid contact between the two vocal folds Orloff and Goldman [174], injected botulinum toxin into the vocalis muscle (thyroarytenoid muscle) aiming to provide much needed rest and recovery time to the abused vocal fold tissue.

Briefly, botulinum toxin is a very toxic peptide protease that binds to nerve endings at the point where the nerves join muscles [174]. This blocks the release of acetylcholine and thereby prevents the nerves from signaling the muscles to contract. The result is weakness and paralysis in the injected muscle. They also reported that the inhibitory effects of botulinum toxin are gradually overcome as axonal outgrowth, which occurs by collateral and non collateral sprouting, leads to reinnervation. It was observed

that botulinum toxin injection induced rest period helped cure vocal granulomas and prevented recurrence in patients who had previously reported instances of lesion recurrence post surgical excision. Orloff and Goldman recommend the use of botulinum toxin (Botox A; Allergan Inc, Irvine, CA) in combination with excisional surgery and voice therapy to yield optimum results. One of the proven disadvantages of botulinum toxin injections into the vocal fold tissue is the potential to permanently alter a patient's voice [174]. Evaluation of observations mentioned above must be carried out bearing in mind that botulinum toxin is one of the most poisonous substances known to mankind. The above study suggests no dosage limits and advocates multiple injections to achieve complete muscular relaxation in an attempt to prevent vocal fold apposition. Excessive levels of the toxin may have deleterious effects on other muscles of the body especially the respiratory muscles which could be fatal.

(k) **Mitomycin-C** - Garrett and co-workers, injected an antibiotic mitomycin-C into injured vocal folds in an attempt to cure scarring [175]. The results were not encouraging since the site of injury demonstrated poor healing, reduced number of fibroblasts subsequently leading to a decrease in the collagen concentration. It is hypothesized that mitomycin-C causes a reduction in the number of fibroblasts which leads to reduction in the amounts of protein synthesized [150]. Thereby wound healing was compromised for want of adequate proteins that bring about resolution of wounds.

The above study promotes the understanding that alleviation of scarring associated pathologies can be achieved by stimulating fibroblasts to synthesize the

appropriate amounts of proteins in the appropriate locations and not by reducing the fibroblast cell number.

2.7.3.2 Natural and synthetic biomaterials used for vocal fold augmentation purposes

Physiological limitations of non degradable synthetic materials and theoretical advantages of natural degradable biomaterials have led to the use of materials like collagen, fat, fascia and HA for vocal fold augmentation purposes.

(a) Collagen - Collagen is useful in treating atrophied vocal fold tissue, scarred vocal fold tissue, minimal glottic incompetence, and focal fibrosis [162]. Collagen injections can be placed anywhere in the lamina propria or the vocalis muscle (thyroarytenoid muscle) [153, 176]. These collagen injections (approximately 0.75ml) can be administered with a 26 or 27 gauge needle which allows precise placement in the plane of interest [153, 162]. Ford and co-workers, administered collagen injections by indirect laryngoscopy or direct microlaryngoscopy with topical anesthesia [162].

- **Bovine collagen preparations** - The various forms of available bovine collagen preparations are Zyderm, Zyplast, Phonagel, Koken Atelocollagen, Gelfoam, Collastat, and Avitene [177]. Of these the biological properties of Zyderm, Zyplast, and Phonagel are the most suitable for vocal fold applications. ZCI-collagen (Zyderm collagen implant) :- ZCI-I and ZCI-II (Zyderm, Collagen Corp, Palo Alto, CA) are composed of 35mg/ml and 65mg/ml of highly purified bovine dermal collagen in PBS with 0.3% lidocaine [153]. Zyderm collagen was first used in 1985 [178]. GAX-collagen

(Glutaraldehyde crosslinked collagen) is 35mg/ml bovine dermal collagen crosslinked by 0.0075% glutaraldehyde [179]. GAX-collagen is marketed as Phonagel® (Palo Alto, CA). These collagen preparations contain approximately 95% collagen type I and 5% collagen type II [153]. Zyplast-collagen (Collagen Corp, Palo Alto, CA) is the same as Phonagel but for the lidocaine concentrations [180]. Indication for use was glottic incompetence due to vocal fold paralysis. It was reported that GAX-collagen injections into the vocal ligament [153] as well as the LPd alone [181] were well tolerated. Also, these collagen injections into the LPs do not have any negative effect on mucosal wave pliability [182, 183]. Since 1986, ZCI-collagen has been replaced by GAX-collagen which is advantageous due to crosslinking [181]. Crosslinking provides the following advantages :- (a) heightened insensitivity against collagenase thereby minimizing risk of resorption (b) no loss of injected volume due to in situ consolidation and (c) less antigenicity. Thereby, there is no need for over correction or re-injection. GAX-collagen induces minimal necrosis and inflammation in the surrounding tissues [179]. Both collagen injections into the vocal ligament showed varying degrees of cellular infiltration and minimal inflammatory response [153]. Histological examination after two months of injection reveals a higher degree of both structural homogeneity and cellular infiltration in ZCI collagen than GAX collagen. The reason behind increased structural homogeneity of ZCI collagen is that once injected the saline solution is resorbed and the collagen fibers are arranged into a homogenous mass. Crosslinking in GAX collagen prevents any such volume reduction. Increased resistance to collagenase activity and crosslinking is the reason behind the relatively acellular appearance of the GAX collagen. But Ford and

co-workers, reported a significantly higher degree of cellular colonization of GAX collagen after two months of injection [179]. This discrepancy may be explained in terms of reliance of homogeneity, function, fate, and future of collagen implants upon the location of injection [179]. It must be noted that Remacle and co-workers, injected GAX collagen into the vocal ligament [153], where muscular activity is more in comparison with the LPs which was the location for injection by Ford and co-workers [179]. Increased muscular activity caused the collagen fibers to break resulting in faster resorption. Also, it is thought that when injected into the vocal ligament the collagen fibers may get dispersed due to muscular activity and may migrate into less compact tissue [176]. On the contrary, collagen injection into the LPs minimizes breakdown of collagen fibers and facilitates cellular colonization of the implant. Most investigators have injected collagen into the LPs due to problems of rapid resorption associated with injection in the deeper layers of the vocal mucosa [162]. Also, since the superficial layers of the vocal fold tissue are devoid of lymphatic drainage it contributes to the longevity of the injected collagen [180]. Inherently, GAX-collagen may also impair cellular function to a certain degree due to the presence of small amounts of the glutaraldehyde crosslinker. At time periods of over one year, GAX collagen showed an appreciable increase in cellular infiltration and neovascularization. 3 to 4% of the patients treated with ZCI-collagen showed hypersensitivity reaction whereas only 1% of the patients treated with GAX-collagen showed a similar reaction [184]. Collagen injections have been used to correct irregular free edges of the scarred vocal fold tissues post Teflon granuloma removal [162]. Failure to gain FDA approval due to concerns

regarding immune reactions has limited the number of clinical trials in USA but this technique has been used successfully elsewhere in the world [162].

GAX-collagen yielded good results even after 4.5 years of initial injection [180]. It was widely accepted that GAX-collagen (and later Zyplast) is a better vocal fold tissue bioimplant than ZCI-collagen because of properties like reduced antigenicity, no need for over correction (no loss of volume in situ), increased resistance to collagenase and subsequent long-term stability [153]. These collagen implants come close to fulfilling Arnold's criterion of being easily injectable, long lasting and well tolerated by the host tissue. Collagen is the first material that was aimed at softening the vocal fold scar tissue [162] since it has been shown to reduce dermal scarring [185].

- **Allogeneic collagen preparations** - Alloderm and Dermalogen are cadaveric acellular human dermal (collagen + elastin) preparations which provide advantages of autologous grafts and nullify problems associated with donor site morbidity. The Alloderm and Dermalogen injections vary in terms of modes of preparation and concentrations of collagen and elastin particles [176]. A micronized version of Alloderm called Cymetra (Lifecell ©, Branchburg, NJ) is used for vocal fold tissue augmentation [186]. It is an acellular dermal allograft that can be injected into the vocal fold tissue whose collagen and elastin framework facilitates cellular colonization and neovascularization. Unlike bovine collagen preparations, a test injection is not necessary for delayed allergic reaction assessment. Though long-term data is unavailable, Cymetra is believed to provide better long-term biointegration than bovine collagen preparations [41]. Alloderm based augmentation of the vocal fold tissue shows peak cellular

(fibroblast) infiltration at day 4 which declines at day 7 and levels off by day 30 [176]. Contrastingly, Dermalogen (Collagenesis, Beverly, MA) [18] based injectables showed peak cellular infiltration at day 30 which eventually leveled off by day 60 [176]. The Dermalogen graft remained stable until day 180 when fat cells were observed peripheral to the graft and neovascularization was detected within the graft. The Alloderm and the Dermalogen grafts became progressively more compact by day 7 due to resorption of water. Unlike what was mentioned earlier, Alloderm and Dermalogen based collagen injections into the vocalis muscle (thyroarytenoid muscle) caused a change in the normal vibratory patterns of the vocal folds. Allogeneic cadaveric collagen preparations last twice as long as bovine collagen injectables. The intact telopeptides in allogeneic collagen preparations increase resistance to proteolytic degradation.

(b) Autologous fat - Autologous fat implantation for vocal fold tissue augmentation is a safe technique since neither short term nor long-term complications have been reported till date [187]. Autologous fat injections are directed into the vocalis muscle (thyroarytenoid muscle), with overinjection to correct for loss of bulk due to resorption. One must be careful to avoid injection into the subglottic space. Indication for fat injection is unilateral paralysis. The first reported autologous fat injection (1991) was performed in the vocalis muscle (thyroarytenoid muscle) of 3 patients, the location being similar to that of Teflon injection [188]. Sataloff and co-workers, performed autologous fat implantation in a pocket created above the scarred tissue along the vibratory margin and co-workers [36]. Both these techniques yielded improved voice intensity, lower phonation threshold pressures and better stability in terms of acoustic output. It is

observed that 40% of the fat cells survive for approximately a year [13]. The unpredictable nature of fat resorption entails overinjection/overcorrection and also re-injections. Long-term studies have been unable to ascertain whether the fat cells in the graft were from the host or surviving cells from the initial injection. Studies by Shindo and co-workers, suggest that injected autologous fat does survive for 6 months but the intensity of voice is lowered after the first 3 months which is indicative of loss in tissue bulk due to resorption [187]. Autologous fat graft survival depends upon the survival of fat cells which in turn is contingent upon the harvesting process, microinjection technique (18 gauge needle) and mechanical stress [187, 189]. Purification process results in reduced fat graft survival. During purification, the fat cells are subjected to homogenization which destroys the fibrovascular scaffolding of the fat cells thus making neovascularization of the graft more difficult [189]. The two fat cell processing techniques are :- (a) morselization, centrifugation and aspiration of resulting pure fat cells and (b) liposuction. It is observed that the liposuction derived fat cells had better survival rates [190]. Since there are no adverse side effects, fat injections may be performed as a temporary procedure to improve vocal performance until a permanent procedure like thyroplasty or arytenoid adduction is performed [187]. The size of the autologous fat cells depends upon the body mass index (BMI) [189]. Smaller fat cells maintain graft volume better than large spaced out fat cells i.e. large fat cells are resorbed faster. The rate of fat resorption and decrease in graft bulk volume depends upon the size and density of fat cells and matrix around it. Fat injections satisfy Arnold's criterion only partly since they are resorbed fairly quickly.

(c) **Autologous fascia** - Injection augmentation with collagen and fat posed problems associated with reduction of bulk volume due to resorption. Since the early 20th century fascia had been used for facial surgery but only in 1998 was its application extended to vocal fold tissue augmentation [191]. Fascia implantation was first introduced in 1998 for curing glottic incompetence [191]. Indications for fascia injection augmentation are glottic incompetence due to recurrent laryngeal nerve paralysis. Fascia can be easily harvested and is very stable. Fascia comprises mainly fibroblasts interspersed in a collagen matrix. Similar to autologous fat, fascia injections were placed in the vocalis muscle (thyroarytenoid muscle). Insufficiencies arising out of unilateral vocal fold paralysis require only very small amounts of space filling materials. Thereby, chopped autologous fascia injections which do not enhance tissue 3D bulk significantly can be used to ameliorate minimal glottic incompetence. Unlike autologous fat injections, fascia injections are not over corrected. Care is taken to prevent fascia extrusion into the vocal mucosa. Since the metabolic requirements of the fibroblasts in fascia tissue are low the free fascia graft can survive as a living tissue. Fascia processing techniques can affect the physiological viability of the fascia to be implanted [141]. The procedure for fascia injection is short and is done as an outpatient procedure. It is assumed that autologous fascia is free of infection and immunologically safe [141]. Long-term (3 years) results suggest that fascia injections are infection free, immunologically safe, bring about an increase in maximum phonation time. Temporal fascia is highly suitable for transplantation into the Reinke's space (LPs). Even though fascia is not considered to add to 3D bulk as mentioned earlier, long-term results showed a restoration of the vocal

fold tissue bulk. Based on the above observations it has been hypothesized by Tsunoda and co-workers [141] that fascia implantation facilitates regenerative-type repair of the vocal mucosa by a mechanism similar to stem cell transplantation in other organs.

(d) Autologous cartilage - Caballero and co-workers, conducted studies on rabbit models and suggested that implantation of autologous auricular cartilage by thyroplasty type I is desirable for correction of vocal impairments arising out of incorrect tissue geometry [192]. Lee and co-workers, extended the use of auricular cartilage for vocal fold augmentation injections [193, 194]. They reported that a mixture of minced auricular cartilage and autologous fat injected 3 to 5mm deep into the vocalis muscle (thyroarytenoid muscle) had a very high survival rate and allowed correction of glottic incompetence even after 3 years. No foreign body reaction (granuloma formation) was reported. Also, histological evaluation revealed no significant differences in implant morphology, and host tissue reaction between 1 and 3 years. There was no evidence of implant resorption since the implant volume remained the same even though the chondrocytes seemed to have lost viability and there was some evidence of fibrosis around the implant.

It seems counterintuitive that the presence of equal amounts of fat and auricular cartilage (elastic cartilage) resulted in the implant maintaining its 3D bulk for 3 years since it has been widely reported that resorption is the single largest reason for autologous fat injections failing in the context of vocal fold tissue augmentation.

2.7.4 Tissue engineering approach for regeneration of the vocal fold tissue

Extensive literature review suggests that voice therapy and surgical augmentation are the primary methods for treating vocal fold scarring and subsequent dysphonias. Although effective in improving vocal quality, neither technique achieves regeneration of native tissue ultrastructure and composition or complete restoration of normal phonation. The techniques discussed earlier were mainly aimed at curing glottic insufficiency secondary to vocalis muscle atrophy due to unilateral vocal fold paralysis, and bowed vocal folds. However, the materials based approach of these techniques encouraged detailed discussion. Ensuing discussion focuses mainly on the materials used or proposed to be used to bring about regenerative-type repair of the human vocal folds. Some of these materials are injected/proposed for injection into the vocal mucosa (LPs) in an attempt to soften scar/prevent scarring, and thereby realize regenerative-type wound healing in the vocal folds. Restoration of the native tissue matrix composition through these methods may facilitate restoration of the normal phonatory voice.

(a) **Hyaluronic Acid (HA)** - HA is widely accepted to be the key molecule that regulates vocal fold tissue viscoelasticity [7]. It has been demonstrated by Chan and co-workers, that particular concentrations of HA have viscoelastic properties similar to that of the human vocal mucosa and that removal of HA from the vocal fold tissue matrix composition by way of injury leads to alteration in tissue viscoelastic properties secondary to scarring [195]. Dahlqvist and co-workers, reported results from a comparative study which involved injecting rabbit vocal folds with HA and collagen separately [38]. It was

observed that the viscoelastic properties of HA injected rabbit vocal folds very closely approximated those of the normal tissue.

Hallen and co-workers, injected a mixture of dextranomer microspheres in a sodium hyaluronan solution (carrier) (DiHA) into the vocalis muscle of rabbit vocal folds [196]. Both dextran based microspheres and HA are non immunogenic. Indications for DiHA injections include unilateral vocal fold paralysis and bowed vocal folds. It was observed that the carrier was resorbed within the first week whereas the dextran microspheres persisted within the tissue 6 months after implantation. Histological evaluation of excised tissue 6 months after implantation revealed that the dextran microspheres were embedded in endogenous connective tissue comprising mainly collagen. These microspheres acted as an attractant for fibroblasts which subsequently produced collagen thereby resulting in soft tissue augmentation. In 2001, Hallen and co-workers, injected DiHA into the vocalis muscle of human subjects who presented insufficient glottic closure arising from unilateral vocal fold paralysis and bowed vocal folds [197]. In some patients, multiple injections were necessary due to resorption of the carrier. It was reported in this study that over a period of 12 months the physical aspects of voice as well as perceptual voice assessment showed considerable signs of improvement.

In 2002 Hertegard and co-workers, used Hylan B gel (Hylaform®, Genzyme, Biosurgery Inc.) to improve the laryngeal function of patients presenting laryngeal dysfunction arising out of unilateral vocal fold paralysis [40]. Hylan B gel is a pure HA gel crosslinked using Michael type addition reaction with divinyl sulfone. Hylan B gels

retain the non allergic, non antigenic and non inflammatory properties of native HA. This study involved comparison of results obtained by Hylan B gel injections to those from bovine collagen injections. Both these materials were injected into the vocalis muscle (thyroarytenoid muscle). A 20% correction was made for collagen injections [180] as compared to a very small overinjection performed for the Hylan B gels (since the resorption characteristics were unknown) [40]. Previously, the same group had shown that the viscoelastic properties of augmented rabbit vocal folds tissue closely approximated those of the normal rabbit vocal fold tissue [198]. Periodic patient assessment (1, 6, and 12 months after injections) revealed improvements in phonation time parameters, glottic closure, and patient's perceptual assessments of voice. Resorption of the injected Hylan B gel was minimal in the first 6 months with no further resorption between months 6 and 12. The regeneration of collagen fibers based connective tissue seemed to be similar to those observed by Hallen and co-workers [39]. This group proposes a 2 year study in the future to assess the long-term resorption characteristics of Hylan B gel.

Finck and co-workers, injected esterified HA (Merogel) into the vocal mucosa (LPs) after surgical removal of benign lesions caused by Reinke's edema [199]. Post removal of the edematous lesion a flap was created in the LPs by microdissection within which esterified HA fibers were placed and then fibrin glue was used to seal the opening. The main reason behind using esterified HA was to ensure that the material degrades with time. The injection was made in the LPs of human patients to allow a physical barrier between the submucosal BMZ and the vocal ligament which would thereby provide

optimal healing conditions for the LPs. Briefly, the pliability of the mucosal shear wave which underlies the quality of voice depends upon the matrix composition of the vocal fold tissue. The purpose behind using esterified HA and not any other material is that HA plays a vital role in eliminating scar formation and its elevated levels during the acute stage of injury (immediately post surgery) would facilitate regenerative-type repair. This material is resorbed within 3 to 4 weeks. The patients reported improved perceptual voice assessments and better phonation times. Laryngoscopic examinations revealed improvements in vibrational amplitudes, glottic closure, motion of the mucosal wave, and no inflammation. The study did not report any histological analysis since implantation procedures were performed in human subjects, each one of which reported alleviated vocal pathology and perceptual voice improvements. A major shortcoming of the above study was the lack of a control group in the absence of which obtained results cannot be interpreted correctly. Evaluation of matrix composition of the endogenously formed tissue could be of value. Assessment of the host/soft tissue response to the implanted esterified HA fibers and their in vivo degradation products may provide valuable insight about the efficacy of this material in vocal fold wound healing. It is widely accepted that polymeric HA is non inflammatory. But one may tend to infer that degradation of high molecular weight HA to lower molecular weights may lead to an inflammatory response. An inflammatory response may ultimately lead to increased collagen deposition following fibroblast recruitment. Restoration of normal voice secondary to regeneration of the LPs using exogenous HA suggests mimicking conditions of fetal wound healing to

achieve regenerative-like repair which is the “holy grail” for tissue engineering researchers.

The HA injections and implants were found to be non toxic and free of foreign body reactions. Minimal inflammation was observed in some patients after the administration of Hylan B gels. The main difference between Hylan B gels (Hylaform) and esterified HA (Merogel) is that the Hylan gels are crosslinked, injectable liquids whereas the Merogel is in the form of solid fibers. Also, Hylan B gels are injected into the vocalis muscle (thyroarytenoid muscle) while the esterified HA is implanted into the LPs.

Hansen and co-workers, have manipulated HA into injectable forms in an attempt to aid vocal fold wound healing and to prolong residence in the tissues to help maintain optimal viscoelasticity of the matrix [43]. The two injectable forms of HA used by this group as proposed materials for alleviation of vocal fold tissue scar are:- (i) HA-DTPH-PEGDA (thiol derivatized HA such that the free thiol groups can react and crosslink with polyethylene glycol diacrylate (PEGDA)), (ii) Carbylan-SX (carboxylic acid groups on HA are modified using carbodiimide chemistry to yield free thiol groups which facilitate crosslinking). These crosslinked materials (crosslinking increases the tissue residence time to approximately 2 weeks as opposed to 3 to 5 days with native HA) were aimed for injection into the vocal mucosa [43]. Klemuk and Titze, compared the rheological properties of ZCI-collagen, Cymetra, and HA-DTPH-PEGDA [200]. It was reported that the viscoelastic properties of HA-DTPH-PEGDA very closely approximate those of the human vocal mucosa. Prior to implantation, rheology revealed that Carbylan-SX had

higher levels of elasticity and viscosity than HA-DTPH-PEGDA [43]. These materials were injected into rabbit vocal folds following a biopsy injury. Saline treated contralateral vocal folds formed the controls. The animals were euthanized after 3 weeks and the vocal fold tissues were subjected to rheology and ELISA. The extent of healing and the effect of injected HA on tissue biomechanics were assessed by rheology. Rheology revealed significant viscosity improvements in tissues injected with HA-DTPH-PEGDA. However, elasticity of the tissues treated with HA-DTPH-PEGDA was similar to that of the control group. In contrast, both elasticity and viscosity were markedly improved in tissues treated with Carbylan-SX in comparison with the saline treated control group. ELISA revealed similar HA levels in all the three groups (HA-DTPH-PEGDA, Carbylan-SX and saline treated control groups). The authors hypothesized that similar levels of HA detected by ELISA (after 3 weeks of implantation) was due to complete degradation of the scaffold material within 2 weeks. Also, since Carbylan-SX is less readily degradable than HA-DTPH-PEGDA it may contribute to maintaining superior viscoelastic properties of the vocal fold tissues post 3 weeks. HA-DTPH-PEGDA may have degraded earlier than 2 weeks (acute phase of wound healing) thus leading to degraded levels of HA similar to scar based wound healing of the vocal fold tissue post injury. This is confirmed by histological evaluations which indicate increased fibrosis in the HA-DTPH-PEGDA groups in comparison to Carbylan-SX groups. Thus it was inferred that Carbylan-SX is a potential injectable material for amelioration of scar tissue based vocal impairments.

From the above observations one may infer that a long-term time course study could lead to better evaluation of the soft tissue response to HA degradation products since it is well known that oligomeric HA is pro-inflammatory. Use of a porcine, canine or sheep models may facilitate assessment of the physical aspects of sounds produced (for example voice intensity) because a rabbit does not vocalize effectively.

(b) Cell based regenerative approach - This is a regenerative approach aimed at alleviating vocal impairments by altering the matrix composition of the tissue thereby improving its functional outcome.

- **Fibroblasts** - Vocal fold scarring is the main indication for injection augmentation with autologous fibroblasts [201]. The fibroblasts are expected to bring about a regenerative-like repair by reconstituting the matrix of the lamina propria in the site of scar and thus influence restoration of normal mucosal wave motion [201]. Autologous fibroblasts are harvested from the patient by skin or mucosal biopsy, and then expanded in culture. The fact that injected fibroblasts do not migrate away from the site of injection is encouraging. It was reported that the administration of three weekly injections of fourth, fifth and sixth passage mucosal fibroblasts (95% – 100% confluence) into the scarred lamina propria (CO₂ laser induced scar) of 9 beagle dogs (animals lack vocal ligament) resulted in restoration of the mucosal wave [201]. The laser-induced injury caused re-epithelialization but not regeneration of the damaged tissue resulting in partial or total loss of the mucosal wave prior to fibroblast injections. Chhetri and co-workers, inferred that since fibroblast injections do not bring about appreciable alteration of the tissue 3D bulk volume, the favorable functional outcome was a direct result of a

replenished lamina propria [201]. Histological studies do indicate presence of native matrix components but in aberrant concentrations. In fact histology indicated an increase in the densities of collagen fibers and a corresponding decrease in elastin. These observations are similar to those of a scarred vocal fold tissue [150].

From the above observations one might infer that the study has scope for further advancements. It seems counterintuitive that though the technique is aimed at eliminating the ill-effects of a scar tissue, therapy itself could lead to a more matured scar in time to come. It is widely accepted that the cell density in the lamina propria is very low. In this study each weekly injection involves introduction of 25 million mucosal fibroblasts (suspended in 0.8 – 1ml of PBS) into the otherwise paucicellular lamina propria. This is a very high cell density which may contribute to the irregular amounts of matrix components detected. The study was limited in the context of measuring acoustical parameters like maximum phonation time, and dysphonia due to the use of a canine model. Hence all observations are based on the videostroboscopic analysis of the mucosal wave which may be inconclusive. No rheological data of the post mortem beagle vocal fold tissues was provided to back up the claim that the fibroblast injection helped to restore tissue viscoelastic properties. Future publications from this group addressing issues like long-term results in human subjects could be really interesting.

- **Mesenchymal stem cells (MSCs)** - It is a widely accepted fact that stem cells can differentiate into a variety of cell types depending upon the epigenetic stimuli they are subjected to. In 2003, Kanemaru and co-workers, injected mesenchymal stem cells into injured vocal folds of a canine model and reported observations on wound healing in

terms of morphological appearance and histological composition [202]. This study comprised vocal fold tissue (animals lack vocal ligament) injection of bone marrow derived stem cells dispersed in a 1% HCl atelocollagen scaffold. This is the first attempt at vocal fold regeneration consistent with the tissue engineering approach. The mesenchymal stem cells were autologous, thereby eliminating fears of immunological safety and ethical issues [202]. Four days post MSCs injection, the vocal fold tissue was damaged with an electrocautery with the injury extending into the vocalis muscle in some cases. Morphological examinations evaluating vocal fold surface irregularity and 3D tissue bulk volume reveals that regeneration of the experimental group (MSCs injection) was more encouraging than that of the control group (1% HCl atelocollagen i.e. scaffold injection). Histological evaluations suggested that apart from cellular nuclei staining in the experimental group (due to the presence of MSCs) there was no significant difference between the control and the experimental groups. Tissue engineering aims to bring about organ regeneration by manipulating the interaction between variables like the scaffold, cells, regulating factors (exogenous/endogenous growth factors), and the appropriate epigenetic stimuli [203-206]. From what was reported [202], the above study did not include any exogenous growth factors like HGF or TGF- β 1. It was reported that unlike the left vocal fold there were no atrophic changes in the right vocal fold [202]. It seems counterintuitive that despite marked differences in the bulk volume and tissue appearance of the MSCs injected tissue, histology did not reveal any significant difference in terms of matrix composition. It seems unlikely that MSCs injection could be causing the space filling effect in the experimental group.

One could interpret these findings in terms of glottic incompetence arising in a manner similar to unilateral vocal fold paralysis. In unilateral paralysis motion of the mucosal wave of both the vocal folds is partially or completely impaired. A similar situation may be arising in this study wherein glottic incompetence due to atrophy in the left vocal fold may be causing reduction of the mucosal wave in both the vocal folds thus eliminating the epigenetic stimuli regulating the unique vocal fold tissue matrix composition. This may be the reason behind no appreciable difference in the matrix composition of the right and left vocal folds even though the right vocal fold tissue morphologically appeared to have a much higher degree of regeneration than the left (MSCs dispersed in a 1% HCl atelocollagen scaffold was injected into the right vocal fold and the scaffold alone injected into the left vocal fold).

(c) **Growth factor therapy** - It is a widely accepted fact that growth factors are potent regulators of cellular functions. Growth factors have been identified as one of the most potent factors regulating cellular function in the vocal fold tissue [49]. Exogenous application of growth factors to repair tissue injuries may require multiple injections to maintain physiologically relevant levels of the growth factor in order to compensate for losses due to rapid metabolization.

- **Hepatocyte growth factor (HGF)** - It has been reported that HGF is responsible for prevention of scarring in the lungs, liver, and kidneys of animal models [207]. HGF is a potent mitogen for mature hepatocytes and is a multifunctional polypeptide involved in embryogenesis, angiogenesis and tissue repair [207]. However, the role of HGF in vocal fold wound healing is not well understood [208] though HGF has been implicated in

suppressing expression of TGF- β 1 and stimulating protease activity that is responsible for degradation of the excess matrix laid down in case of scar formation [207]. HGF activity in acute vocal fold tissue wound healing of rabbits was shown to be activated by day 10 and lowered slightly by day 15 [207]. In case of wound healing in artificial wounds created in a rat model, the mRNA levels of HGF increased steadily from day 1 to day 14 [209]. Contrastingly, peak mRNA expression levels of HGF was reported at day 3 of epithelial wound healing [210]. These observations suggest that HGF activation depends upon the site and the extent of tissue damage. Briefly, it is hypothesized that the mechanism of action of HGF is paracrine [207], in that the fibroblasts in the lamina propria produce HGF which is then transferred to epithelial cells with receptor c-Met[208]. Once the HGF is bound to the c-Met it is said to modulate interactions between stromal epithelial cells associated with scarring. Autocrine production of HGF by epithelial cells is neither proven nor has it been ruled out. Thus, HGF functions to regenerate the epithelial cells through stromal-epithelial interactions [48].

Hirano and co-workers, have demonstrated that HGF upregulates HA synthesis and downregulates collagen synthesis by both canine and human vocal fibroblasts in vitro thus strengthening claims that HGF prevents scar formation [46]. In another study, the same group injected HGF into rabbit vocal folds to evaluate the effect of HGF on scarring [48]. The procedure involved unilateral vocal fold stripping, immediately followed by HGF administration into the injury site. In this study HGF was administered only once in the beginning of the study.

A similar study was undertaken by Hirano and co-workers, to study the therapeutic effects of HGF injections on canine vocal fold tissue scarring [47]. In this study, HGF was injected into the vocal fold tissue one month post induction of scarring by stripping. Histological examination of tissues (from both the above studies) 6 months later showed a reduction in both elastin and collagen deposition (precursor to scarring) and a corresponding reduction in tissue contraction (onset of scarring) in comparison to the untreated control group. Though the vibratory and viscoelastic properties of the treated tissue were better than the untreated control group the phonation threshold pressure and physical aspects of voice were still below normal levels.

One might infer that HGF should be introduced into the injury site as early as possible to coincide with the onset of the proliferative stage of wound healing. Rapid degradation in the levels of HGF may necessitate multiple injections.

- **Basic fibroblast growth factor (bFGF)** - It has been reported that bFGF plays a vital role in upregulating HA synthesis and downregulating collagen synthesis by aged rat fibroblasts [211]. As we age, the collagen levels in our vocal fold tissue increase (due to reduced rate of ECM turnover) and HA levels decrease leading to dysphonia secondary to increased stiffness and altered viscoelastic properties of the vocal fold tissue. Hirano and co-workers, attempted to reverse negative changes in the matrix protein levels leading to presbyphonia by administering bFGF 4 times weekly in a time course study spanning 2 months [49]. At the various time points (1 week, 1 month, and 2 months) there was a consistent increase in the HA levels of the bFGF treated tissue in comparison with untreated tissue (control group). But comparison of HA levels across the 3 time points

revealed that the HA levels in treated vocal folds was significantly reduced 2 months after injections as compared to that after 1 week. Collagen levels remained similar in both the bFGF treated tissue and the untreated tissue across all the time points.

From the above observation, one may infer that therapy based on bFGF delivery may require re-injections to bring about a favorable outcome. On the flipside, exogenous growth factor injections result in their rapid metabolization in tissues before the desired effect is achieved in most cases. In the aforementioned study, this may be a reason behind unchanged collagen levels despite repeated injections. In aged subjects, depolymerization of collagen is time consuming since protease production by senescing fibroblasts is less efficient. Rapidly degrading levels of exogenously applied growth factors like bFGF and HGF therefore do not produce the desired stimulatory effect on senescent fibroblasts.

The above study may incorporate the use of a canine model or even human patients and then subjecting the excised tissues to rheological tests to see how well the viscoelastic properties of the treated vocal fold tissue approximate those of the non pathological native tissue. Motion of the mucosal wave should be assessed by videolaryngostroboscopy. In case of human subjects, where tissue excision is not a possibility, the physical aspects of voice like phonation threshold pressure, maximum phonation time, vocal efficiency, and voice intensity should be assessed.

Use of gene therapy or controlled release methods which may maintain elevated growth factor levels in the tissue will enhance the efficacy of delivered growth factors in vivo [49]. However, in the context of HGF and bFGF based attempts at vocal fold

regeneration, one must bear in mind that there exists no example of single growth factor delivery mediated alleviation of pathology.

(d) ECM scaffold - Porcine-derived xenogeneic ECM scaffolds have been used to bring about repair and reconstruction in numerous animal and human studies [212]. These porcine-derived scaffold materials maintain their 3D ultrastructure due to the presence of fibrous proteins like collagen types I, III, IV, V, and VI, structural glycoproteins like fibronectin, GAGs, growth factors like VEGF, TGF- β 1 and bFGF [212]. These ECM based scaffolds are acellular and so cell-mediated immune response is minimal if any. Moreover, any persistent antigenic response is eliminated by the rapid degradation of the scaffold. Head and neck reconstruction procedures and surgeries to alleviate laryngeal cancer are indications for use of porcine derived ECM scaffold for laryngeal reconstruction. In the time course study reported by Huber and co-workers [212], the entire vocal fold tissue and 70% of the thyroid cartilage of a canine model were surgically excised and replaced anatomically by the porcine-derived ECM scaffold as correctly as possible. Interestingly at all time points beyond 3 months to 1 year, presence of collagenous connective tissue with submucosal glands with an overlying intact squamous epithelium marked the remodeling of the scaffold material.

One might hypothesize that either the scaffold showed good biointegration with the native host tissue or the scaffold had been remodeled completely by host tissue since it was reported that the boundary between the scaffold and the host tissue was invisible after 3 months. This study claims that microstructure and macrostructure of the newly formed tissue was similar to that of the original tissue. Since this review is focused on

the membranous vocal fold tissue, it must be pointed out that the canine vocal fold tissue not only lacks the vocal ligament but also possesses a dense collagenous lamina propria. This is vastly different from the composition of the human vocal fold tissue. Therefore successful porcine-derived ECM based reconstruction of the 3D anatomic structure of the human vocal fold tissue may not yield positive results to the extent reported in this study.

Also, this study aimed only to obtain an acceptable speaking voice in patients who have lost phonatory ability due to surgical removal of lesions. It thereby clarifies that restoration of normal voice was not a priority. Future long-term studies involving human subjects post surgeries for removal of benign or cancerous lesions may tell us a lot more about the ability of the human body to remodel the porcine-derived ECM scaffold into functional vocal fold tissue. Another advantage of conducting such studies on human subjects is that the physical aspects of voice such as voice intensity, maximum phonation time, and fundamental frequency of phonation can be assessed.

(e) **Collagen-HA and collagen-alginate hydrogels** - From the observations, above one could infer that due to problems associated with resorption and allergic response an alternative to allogeneic and bovine collagen preparations needs to be found. Since maintenance of tissue geometry is critical for production of normal voice it is important for injectables to be able to maintain their 3D structure relatively intact in situ even when being remodeled by the host or the encapsulated cells if any. Hahn and co-workers, have reported the advantages of collagen-alginate hydrogels over collagen-HA hydrogels which could be potentially useful for restoring normal matrix composition of the vocal fold tissue in the future [44]. Briefly, this group encapsulated vocal fold fibroblasts in the

collagen-HA as well as collagen-alginate hydrogels, and performed a time course study in order to evaluate cell density and morphology, matrix (collagen and GAG synthesis) production, lateral gel compaction and mass loss. After 28 days of culture, the collagen-HA hydrogels showed extensively interconnected fibroblasts whereas those in the collagen-alginate hydrogels were spindle shaped with few interconnections. The collagen-HA hydrogels showed significant mass loss (50%) associated with gel compaction between weeks 4 and 7 with little evidence of matrix deposition (collagen fibers and GAGs). Contrastingly, the collagen-alginate hydrogels resisted compaction and mass loss for at least 6 weeks (last time point) in culture while facilitating ECM deposition (elevated levels of collagen and GAGs in comparison to week 4). Interestingly, the matrix protein levels detected in collagen-HA gels after 7 weeks were comparable to those in porcine vocal fold tissue. Thus the collagen-alginate hydrogels appear to be promising materials for vocal fold restoration.

From the above observations, one might hypothesize that the collagen-alginate hydrogels are promising potential scaffolds for vocal fold tissue regeneration. It must be pointed out that there exists a slight discrepancy in the assessment of cell density in the 2 hydrogel types at the end of week 4. It was reported that there was no significant difference in cell density from day 0 to week 4. But the BrdU stain showed positive results for the collagen-HA hydrogels, which indicates uptake of the stain by cells in the “S” phase of the cell cycle which is when DNA duplication occurs. Cell proliferation leads to heightened cell migration, and increased cellular interconnections within the scaffolding (personal observations) resulting in heightened contractile forces exerted by

the fibroblasts which explains the compaction of the collagen-HA hydrogels. A negative BrdU stain in the collagen-alginate hydrogels corresponds to a lack of cell proliferation (and therefore less cell migration) which explains single spindle shaped cells without any interconnections eventually leading to maintenance of original gel dimensions even after 6 weeks of culture. One might hypothesize that a DNA quantitation analysis may be performed for additional confirmation of the results previously obtained. In vivo studies using canine models may provide additional insight into the performance of these collagen based composite hydrogels in environments where hydrogel degradation rates could be heightened due to elevated levels of proteases (in case of inflammation) and macrophage activity.

CHAPTER 3

PROJECT RATIONALE

3.1 Hypothesis

The techniques/approaches described earlier have been unable to achieve complete restoration of voice quality due to their inability to bring about regeneration of the native vocal fold tissue architecture/ECM composition. Our long-term hypothesis is that rapid restoration of the mechanical microenvironment using mechano-mimetic scaffolds will minimize scarring, facilitate vocal fold tissue-specific ECM production, and ultimately restore normal phonation. The long-term objective of our research is to prevent vocal fold scarring following injury, achieve regeneration of the vocal fold tissue architecture/ECM composition and thereby restore normal phonation through the use of mechano-mimetic scaffolds. Consistent with the tenets of tissue engineering we aim to use 3D, biodegradable, polymeric scaffolds; in vivo vocal fold tissue manipulation with which will yield biomechanically optimal functional results. We believe that minimally invasive therapeutic intervention/manipulation of the acute stage of vocal fold tissue injury using mechano-mimetic materials could potentially ameliorate vocal fold tissue scarring sequelae and restore long-term normal phonatory voice. We aim to target the acute stage of a vocal folds injury (which leads to the onset of fibrosis) by injecting :- (a) HA-based hydrogels (aimed at restoring viscous shear properties of the vocal mucosa), (b) polyethylene glycol diacrylate (PEGDA) based hydrogels (aimed at restoring elastic integrity of the vocal ligament). Various studies have elucidated the use of in situ photopolymerizable, water soluble macromers with functionalized end groups that can be

crosslinked to realize hydrogels upon exposure to UV light using cytocompatible photoinitiators [213-215]. We opted for HA due to its abundance in the lamina propria, viscoelastic properties, chemical processability, enzymatic degradability, wound healing properties and ability to interact with certain fibroblast cell surface receptors (CD44). PEG has been selected as the starting material among other available synthetic materials due to its FDA approved status, ease of chemical modification, lack of specific cellular interactions and biocompatibility thus providing a “blank-slate” for inducing cell-biomaterial interactions by incorporating synthetic peptides [216]. Observations from our preliminary experiments as well as previously reported studies [213, 217-219] sufficiently indicated that cell viability, proliferation, and ECM production are not adversely affected by UV based hydrogel photopolymerization processes. One must bear in mind that though these in vitro studies dealt with direct UV illumination of the cell-macromer suspensions in order to realize cell encapsulated hydrogel constructs, the efficacy of transdermal injections which assume importance particularly in the context of the proposed vocal folds application has been amply demonstrated by Elisseeff et al [220, 221].

3.2 Specific Aims

Aim 1: To enhance cellular remodeling of PEGDA hydrogels through the creation of PEG based semi-IPNs in order to facilitate improved cell spreading and proliferation in the PEGDA based three dimensional networks. Based on previously published studies [222-224] and observations from our preliminary cell culture

experiments we inferred that the nanometer-scale mesh size of the PEGDA based hydrogel networks restricts encapsulated cells to a rounded morphology that can inhibit cellular processes such as proliferation and migration that are essential for the early stages of remodeling and tissue formation. Hence in the initial phase of the vocal fold regeneration project we aim to investigate an approach for accelerating cellular remodeling based on the creation of semi-IPNs composed of hydrolytically degradable PEGDA macromers and native, enzymatically degradable ECM components (collagen, gelatin, and HA).

Aim 2: To create degradable hydrogels/semi-IPNs approximating the mechanical properties of the vocal mucosa and the vocal ligament that may be photopolymerized in situ following vocal fold injury or surgery using minimally invasive techniques to fill defects. We aim to create mechano-mimetic, degradable, PEG-based semi-IPNs (approximating the elastic stress-strain properties of the human vocal ligament) and GMHA hydrogels (approximating the viscous shear properties of the human vocal mucosa) that may be photopolymerized in situ using minimally invasive methods. We aim to assess cytocompatibility and the ability of these potential scaffold materials to support cell proliferation and neo-matrix deposition by the resident cells.

Aim 3: To assess the ability of fibroblast encapsulated GMHA hydrogels to support vocal mucosa-specific ECM deposition in response to physiologically relevant high frequency vibration. Consistent with our long-term hypothesis, we believe that rapid restoration of the vibratory microenvironment using mechano-mimetic scaffolds will facilitate vocal mucosa-specific ECM production and minimize scarring,

thereby restoring normal phonatory voice in long-term. Towards this end, we aim to assess the ability of the GMHA hydrogels to support human fibroblast formation of vocal mucosa-like ECM in response to vibration. Fibroblast encapsulated GMHA hydrogels will be subjected to physiologically relevant high frequency vibration over a time course, using a custom-built vibrational bioreactor and Labview software.

3.3 Clinical significance

Voice is widely acknowledged as the “mirror of soul” and is significant in terms of the quality of life. Voice is a primary “tool of trade” for 25% of the U.S. work force (teachers, singers, telephone marketers, sales personnel, counselors, etc.) [1, 16]. The quality of voice depends on the biomechanical properties of the vocal fold tissue which derive from its highly differentiated ECM organization and composition. The wound healing response to vocal fold injuries is characterized by scarring and subsequent dysphonia due to alterations in the biomechanical properties of the tissue. Dysphonia characterized by increased effort to sustain phonation, decreased vocal quality, and total voice loss affects approximately 23% of the voice-dependent US workforce [14]; and may jeopardize job security, employment opportunities, and social interaction [225, 226]. Scarring-induced dysphonias are currently treated by voice therapy and surgical augmentation procedures which provide short-term improvement in voice quality. Nevertheless, these techniques have been unable to achieve long-term restoration of normal phonatory voice due to their inability to bring about regeneration of the native vocal fold tissue architecture/ECM composition. The human vocal ligament is abundant

in fibrous structural proteins like elastin and collagen that impart elastic stress-strain properties to the vocal fold tissue. The human vocal mucosa is abundant in HA and proteoglycans that impart viscous shear properties to the vocal fold tissue. The long-term objective of our research is the in situ photopolymerization of autologous cell encapsulated, mechano-mimetic, degradable hydrogels/semi-IPNs to fill defects in specific layers of the human vocal fold tissue following injury. This approach may help restoration of long-term normal phonatory voice by promoting regeneration of native tissue architecture/ECM composition and minimizing fibrotic scarring. Through our research we aim to make a significant attempt to improve human lives that have been negatively impacted by scarring related vocal pathology. **Our contributions to vocal fold tissue engineering are :- (a) the proposed use of mechano-mimetic scaffolds for regenerating the human vocal mucosa (GMHA hydrogels) and the vocal ligament (PEG-based semi-IPNs) in an attempt to restore appropriate mechanical integrity to the vocal fold tissue following injury and (b) the demonstration of the importance of restoring the vibratory mechanical microenvironment in regenerating the unique ECM composition of the native tissue.** In the context of other tissue engineering applications these materials may serve as :- (a) a substrate for host cell infiltration and colonization, (b) a delivery vehicle for autologous cells which will eventually bring about regeneration of the native tissue ECM composition, thereby aiding soft tissue repair.

CHAPTER 4

INCORPORATION OF HYALURONIC ACID INCREASES FIBROBLAST SPREADING AND PROLIFERATION WITHIN PEG-DIACRYLATE SEMI-IPNs

4.1 Introduction

Hydrogels are crosslinked polymer networks that have been widely studied as tissue engineering matrices for cell transplantation and sustained, site-specific delivery of bioactive molecules. Properties such as minimally invasive introduction as aqueous solutions and in-situ gelation, conformation to irregular tissue geometry, high gas and metabolite permeability, and viscoelastic mechanical properties have rendered hydrogels useful for cartilage, bone, vascular, and soft tissue applications [214, 223, 227]. Hydrogel matrices have been prepared from a variety of naturally-derived proteins and polysaccharides, as well as synthetic polymers. Although naturally-derived materials are intrinsically degradable by physiological enzymes, limitations include availability, reproducibility, and limited control over degradation rate and mechanical properties. Hydrogels based on synthetic polymers offer an increased diversity of physicochemical properties that may be tailored in a reproducible manner for specific applications [215]. Degradable synthetic hydrogels have been based on polymers incorporating or modified with hydrolytically susceptible ester bonds, such as poly(ethylene glycol)-polyester block copolymers [228-230], poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)) copolymers [231], and poly(lactic acid)-grafted poly(vinyl alcohol) [232]. Although these synthetic hydrogels are biocompatible, synchronizing the rate of hydrolytic

degradation with cellular growth and matrix accumulation poses a challenge to the realization of engineered functional tissue [223].

At early time points following encapsulation, the 15-35A° mesh size of these highly crosslinked matrices [222] has been shown to restrict cell spreading, resulting in a rounded morphology that can delay cell proliferation, migration, and ECM production [224]. Several approaches have been investigated for increasing cellular activity within synthetic hydrogel matrices. Anseth's group demonstrated that appropriate tailoring of hydrogel degradation rate by mixing macromers of varying chemistry can improve cellular differentiated function and the synthesis and spatial distribution of ECM [233, 234]. Hubbell and West have developed several elegant systems based on crosslinking of non-degradable polymers with synthetic peptides or recombinant proteins by photopolymerization and Michael addition [235-239]. These peptides were designed with reactive terminal groups that formed crosslinks in the polymer network and internal sequences based on cleavage sites recognized by physiological proteases involved in cell migration such as collagenase, elastase, and plasmin. These matrices are chemically stable and undergo localized degradation in response to cellular proteolytic activity. In combination with grafted cell adhesion peptides and incorporation of soluble growth factors, these matrices successfully mimic multiple features of the native ECM and have shown efficacy for in vivo regeneration of bone [240]. One limitation of this approach is the small scale and high production cost of synthetic peptides and recombinant proteins.

Several groups have begun to investigate the utility of biosynthetic composite gels composed of both synthetic polymers and naturally occurring ECM components.

Almany and Seliktar have described a system comprised of chemically crosslinked PEGylated fibrinogen [224]. The fibrinogen component provides bioactivity for cell adhesion and enzymatic degradation, while variation in the content and structure of the PEG component allows flexible control over degradation rate and mechanical properties [241]. Compositions with appropriate degradation rate have been shown to support bone formation in rat tibial defects [242]. Sefton and coworkers have described the development of semi-synthetic collagen/poloxamine matrices that support the viability of encapsulated cells and the adhesion and spreading of various cell types on the gel surface [243, 244].

The objective of the current studies was to investigate an alternative approach for accelerating cellular remodeling based on the creation of semi-IPNs of covalently crosslinked, hydrolytically degradable PEG-diacrylate macromers with native, enzymatically degradable ECM components homogeneously dispersed in the network. Low levels of hyaluronic acid (HA) are shown to facilitate 3-dimensional fibroblast spreading and proliferation through a mechanism of cell-mediated enzymatic degradation.

4.2 Materials & Methods

4.2.1 Materials

Polyethylene glycol (PEG, MW=3934) was purchased from Fluka (Seelze, Germany) and 2-chloropropionyl chloride, triethylamine, dichloromethane (HPLC grade), anhydrous sodium sulfate, and sodium acrylate were obtained from Aldrich (St.

Louis, MO, USA). Dimethyl formamide (extra dry) and ethyl ether were purchased from Acros (Morris Plains, NJ) and Fisher Chemical (Fair Lawn, NJ), respectively. 2-hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone (I-2959) was purchased from Ciba Specialty Chemicals (Basel, Switzerland). Adult normal human dermal fibroblasts (NHDF, CC-2511) obtained from a 39 year old Black woman were purchased from Biowhitaker (Rockland, ME, USA). DMEM F-12 50/50, 1X with L-glutamine, 15mM HEPES/trypsin EDTA (0.05% trypsin/0.53mM EDTA in HBSS), and penicillin/streptomycin were obtained from Mediatech (Herdon, VA, USA). Bovine growth serum was purchased from Hyclone (Logan, UT, USA). Gelatin and collagen (Vitrogen 100) were purchased from Mallinckrodt Specialty Chemicals (Paris, KY, USA) and Angiotech Biomaterials (Palo Alto, CA, USA), respectively. Hyaluronic acid (HA, MW=1.4 x 10⁶) was purchased from Genzyme (Cambridge, MA, USA).

4.2.2 Synthesis of degradable PEG-bis-(2-acryloyloxy propanoate) (PEG-bis-AP) macromers

PEG-diacrylate macromers with hydrolytically degradable ester bonds were synthesized by a two-step reaction as previously described [245].

4.2.2.1 Synthesis of PEG-bis-(2-chloropropanoate), (PEG-bis-CP)

15g of PEG (3.75mmol) was dissolved in 120ml of dry dichloromethane and introduced in a dry, three-neck-250ml flask under inert (argon gas) conditions. Once the PEG was completely dissolved, 0.94ml of triethylamine (6.75mmol) was added to the

three-neck flask which was kept immersed in an ice bath. 1.5ml of 2-chloropropionyl chloride (15mmol) was added to 30ml of dry dichloromethane in a dropping funnel. Contents of the dropping funnel were introduced into the three-neck flask in a drop-wise manner within 2hours under constant agitation. After approximately six hours, the ice bath which was never replenished, was removed and the reaction was allowed to progress overnight at room temperature. The TEA-HCl salt was removed by filtration of the reaction mixture using a buchenel glass funnel (10 – 15 μ m pore size). The filtrate was then reduced to 1/10th of its initial volume using a rotary vacuum evaporator (Buchi Rotavapor®, Switzerland) and precipitated using 10X cold ethyl ether. The precipitate was re-solubilized in 10X dry dichloromethane (150ml) and introduced into a 500ml separating funnel. The product was washed twice with 15ml of 10% NaHCO₃, followed by 3 washes with 15 ml distilled water. At this point the pH of the product was neutral. Residual water was removed by the addition of anhydrous sodium sulfate until the physical state of sodium sulfate changed from clumps to a consistent powder. Once again the dichloromethane was reduced to 1/10th of its initial volume by vacuum evaporation and the product (PEG-bis-CP) was precipitated in 10X cold ether and washed twice in 300ml of ether. PEG-bis-CP was then recovered, weighed, and stored in a dessicator under a continuous vacuum. The structure of PEG-bis-CP and the degree of substitution were determined from the ¹H-NMR (Brucker 300 MHz, CDCl₃) spectra as previously described [246]. The reaction scheme for PEG-bis-CP is shown in figure 4.1.

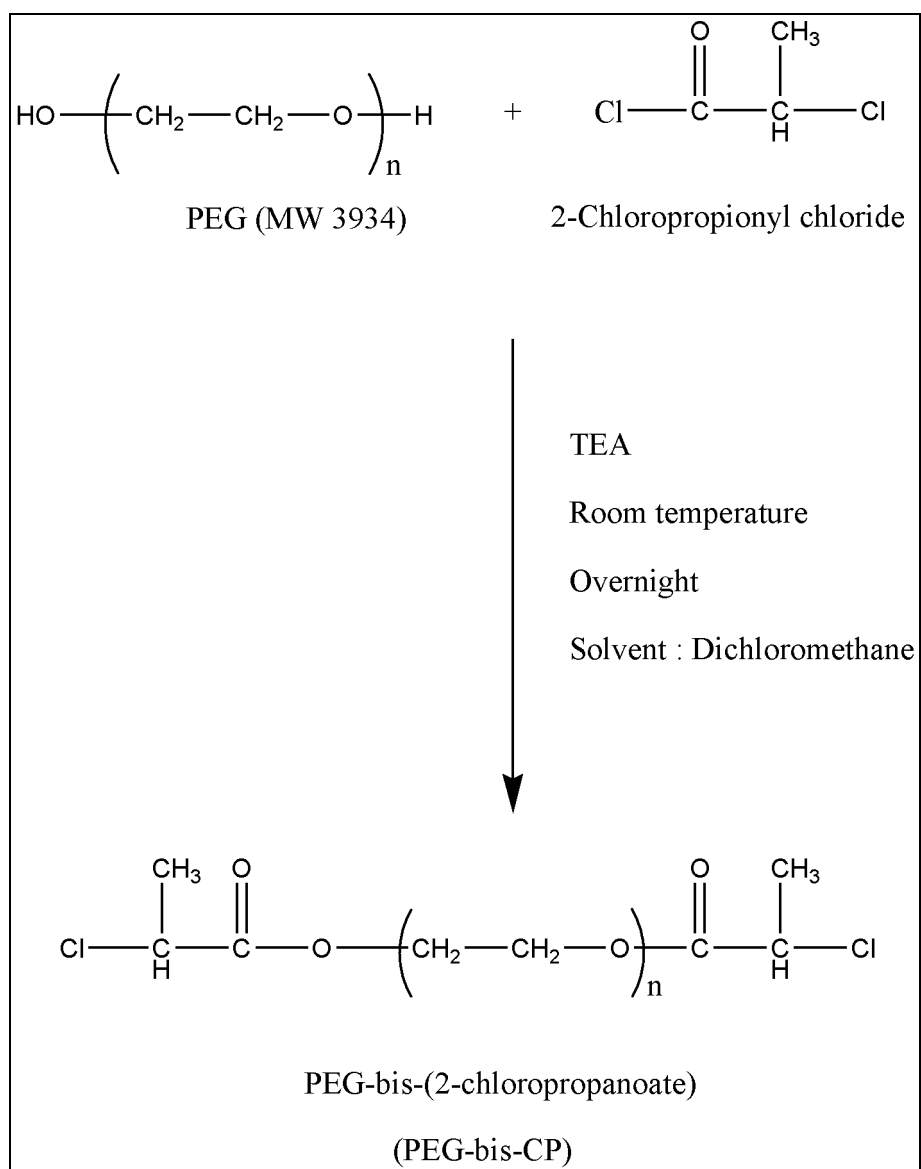


Figure 4.1 Chemical reaction scheme for PEG-bis-CP (intermediate product).

4.2.2.2 Synthesis of PEG-bis-(2-acryloyloxy propanoate), (PEG-bis-AP)

10g of PEG-bis-CP (2.5mmol) was solubilized in a dry, three-neck-250ml flask by the addition of 100ml anhydrous dimethylformamide in the presence of small amounts of inhibitor 4-methoxyphenol. 1.18g of sodium acrylate (15mmol) was added to the contents of the three neck flask under constant agitation in an inert atmosphere (argon gas). The reaction was refluxed for 30 hours at 85°C under inert conditions, followed by cooling to room temperature and filtration of the reaction mixture through a buchenel glass funnel (10 – 15µm pore size) in order to remove unreacted sodium acrylate. The dimethylformamide was reduced to 1/10th of its initial volume by vacuum evaporation and the remaining contents of the reaction were precipitated in 10X cold ether and washed twice in 300ml ether. The product (PEG-bis-AP) was recovered, weighed and stored under dark conditions in a vacuum dessicator. The structure of PEG-bis-AP and the degree of acrylation were determined from the ¹H-NMR (Brucker 300 MHz, CDCl₃) spectra as previously described [246]. The reaction scheme for PEG-bis-AP is shown in figure 4.2.

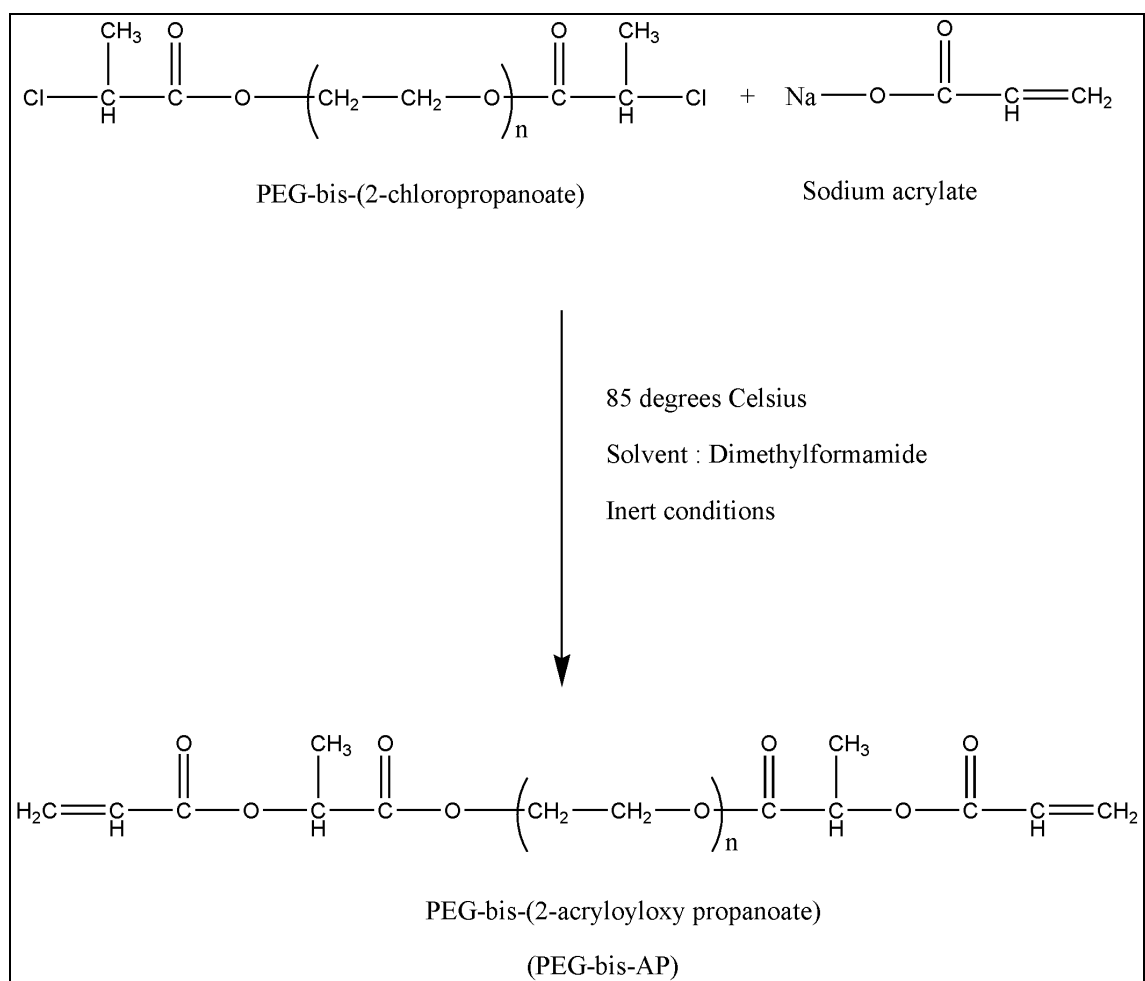


Figure 4.2 Chemical reaction scheme for PEG-bis-AP macromers.

4.2.3 In vitro cell culture

NHDF were routinely cultured in 182 cm² T-flasks using DMEM F-12 50/50, 1X with L-glutamine and 15mM HEPES enriched with 10% v/v bovine growth serum and 50U/ml penicillin and 50µg/ml streptomycin. Culture medium was changed once every two days and cells were passaged weekly. All experiments were performed with cells from the 4th – 6th passages. For hydrogel encapsulation, monolayers of fibroblasts (ca. 95% confluence) were trypsinized, centrifuged, resuspended, counted in a hemacytometer, and adjusted to a final concentration of 1.6×10^7 cells/ml.

4.2.4 Hydrogel/Semi-IPN photopolymerization

PEG-bis-AP macromer solutions were prepared in 1X-PBS (0.1M, pH 7.4) and sterile filtered. A 10% w/v stock solution of I-2959 was prepared in 70% ethanol. A fibronectin-derived cell adhesion peptide, (GRGDS, Bachem, PA, USA) was conjugated to acrylate-PEG-NHS (MW=3400, Nektar) as previously described [216]. GRGDS-PEG-acrylate was prepared as a stock solution in 1X PBS (10 µmol/ml) and sterile filtered. PEG-bis-AP hydrogels and semi-IPNs (final concentration 6% w/v) containing varying types and concentrations of unmodified ECM molecules with 0.1% w/v I-2959 initiator were photopolymerized between glass microscope slides separated by a 1mm Teflon spacer by exposure to low intensity UV illumination (365 nm, 10 mW/cm², Black-Ray B100-AP, Upland, CA) for 7½ minutes per side. For cell encapsulation studies, acrylate-PEG-GRGDS and NHDF were included at final concentrations of 2.5µmol/ml and 4×10^6 cells/ml, respectively.

4.2.5 Effect of various ECM molecules on fibroblast morphology in PEG-based semi-IPNs

NHDF were encapsulated as described above in 6% w/v PEG-bis-AP based hydrogels and semi-IPNs containing equal concentrations (0.12% w/v) of gelatin, collagen, and HA (n = 4 samples/condition). Samples were cultured in 12 well plates for 7 days, and then stained with 1 μ M fluorescein diacetate (FDA, Molecular Probes, Eugene, OR) and 2.5 μ M propidium iodide (PI, Molecular Probes), rinsed with PBS, visualized by fluorescence microscopy (Zeiss Axiovert 200) and digitally imaged.

4.2.6 Effect of HA concentration on fibroblast morphology and proliferation in PEG-based semi-IPNs

NHDF were encapsulated as described above in 6% w/v PEG-bis-AP based hydrogels and semi-IPNs containing 0, 0.06%, 0.12%, and 0.18% w/v HA (n=9 samples/condition). Samples were cultured for 1 – 14 days.

4.2.6.1 Confocal analysis of morphology

After 14 days in culture, 3 samples from each group were fixed in 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO, USA) in PBS, permeabilized with 0.1% Triton X-100 (Sigma), and stained with Alexa 594-phalloidin (Molecular Probes). Samples were visualized and imaged using a Zeiss Confocal LSM510 microscope.

4.2.6.2 Fibroblast proliferation

3 samples from each group were harvested at day 1 and day 14, rinsed twice with sterile PBS, snap-frozen in an ethanol-dry ice bath, and stored at -80°C. DNA solubilization and quantitation were based on modifications of previously described methods [247]. Briefly, the samples were subjected to 3 freeze-thaw cycles in 1.4ml of 10mM EDTA, pH 12.3. Once the hydrogels were completely dissolved, the pH was neutralized by the addition of 100 μ l 1M potassium phosphate (monobasic) solution. Total DNA content was measured using the Picogreen DNA-binding dye (Molecular Probes) and a fluorescence microplate reader (Tecan GENios, excitation: 485nm, emission: 535nm). DNA content was converted to cell number based on a DNA standard curve prepared from serial dilutions of NHDF.

4.2.7 Volumetric swelling ratio

PEG-bis-AP hydrogels and semi-IPNs (6% w/v) containing varying amounts of HA were photopolymerized as described above (n=4/group). Circular hydrogel samples (1mm thick, 8mm diameter) were punched out using a hole punch and equilibrated in distilled water for 24 hours to remove any unpolymerized macromer. Samples were lyophilized and dry weights (W_d) were measured. The gels were immersed in distilled water and allowed to swell for 24 hours in order to record the wet weights (W_s). The volumetric swelling ratio and mesh were calculated as previously described [248, 249].

4.2.8 Elastic Modulus

PEG-bis-AP hydrogels and semi-IPNs (6% w/v) containing varying amounts of HA were photopolymerized and cut into dumbbell shaped samples (gauge length 30mm, gauge width 5mm, and thickness 1mm). The samples (n=4/group) were subjected to 15% strain at 5mm/min using an MTS Synergie 100 (MTS Systems Corporation) at room temperature. Each sample was tested twice to prevent errors due to sample slippage.

4.2.9 Degradation studies

PEG-bis-AP hydrogels and semi-IPNs (6% w/v) containing varying amounts of HA were photopolymerized, equilibrated with water, lyophilized, and weighed (Wd_0) as described above. Samples were incubated with 4ml of 1X-PBS containing 0.01% sodium azide (Sigma) in scintillation vials under shaking at 37°C. Buffer was changed once every two days. At weekly intervals, samples (n=3/group/time point) were collected, washed with distilled water three times, lyophilized, and weighed (Wd_t). Percent mass loss was calculated as $((Wd_0 - Wd_t)/Wd_0) \times 100$.

4.2.10 Contribution of hyaluronidase activity to fibroblast spreading in PEG-bis-AP/HA semi-IPNs

NHDF were encapsulated in 6% w/v PEG-bis-AP/0.12% w/v HA semi-IPNs as described above and cultured in 12 well plates for 48 hours in the presence of the hyaluronidase (Hase) inhibitors neomycin trisulfate hydrate (0.1mM, Sigma, 10mM stock solution in PBS) and L-ascorbic acid 6-palmitate (0.1mM, Sigma, 10mM stock

solution in 70% ethanol) [250, 251]. Controls consisted on semi-IPN samples cultured in routine media without the addition of HAse inhibitors, as well as NHDF cultured on the surface of 12 well plates with and without the presence of inhibitors. Cytotoxicity of the inhibitors was assessed by FDA/PI staining of monolayer cultures. Fibroblast morphology and spreading within semi-IPNs was analyzed by confocal microscopy as described above.

4.2.11 Reverse hyaluronan gel zymography

The effectiveness of the inhibitors was confirmed by reverse hyaluronan gel zymography on two 10% polyacrylamide (Bio-Rad laboratories, Hercules, CA, USA) gels each containing 3.4mg/ml HA. The procedure was adapted from two other approaches previously described [252, 253]. Hydrogel samples were frozen and homogenized in 200µl extraction buffer (20mM TRIS buffer pH 7.5, 0.5% (w/v) Triton X-100, 1% sodium dodecyl sulfate (SDS, Fisher) containing 10µl/ml proteinase inhibitor (Complete Proteinase Inhibitor, Roche, Germany) and kept on ice for 10 minutes. The samples were centrifuged for 15 minutes at 15,000rpm and supernatants were collected. The protein content of the gel extracts was determined by BCA assay (Pierce) and diluted with extraction buffer to a final concentration of 40µg protein/60µl. Pre-stained molecular weight markers (10 – 250 kDa) and 60µl gel extract samples were mixed with 20µl Laemmli's sample buffer [254]. 40µl aliquots were loaded on each gel and electrophoresed under non-reducing conditions. After electrophoresis, the gels were washed in 2.5% w/v Triton X-100 for 30 minutes to remove SDS and then incubated in

the substrate buffers. One gel was incubated in a pH 3.75 substrate buffer (50mM citric acid, 50mM Na₂HPO₄, 0.15M NaCl, 0.02% w/v NaN₃). The other gel was incubated in a pH 7.6 substrate buffer (50mM TRIS, 5mM CaCl₂, 200mM NaCl, 0.02% v/v Brij). The gels were then stained with 0.5% Alcian blue (Anatech LTD, Battle Creek, MI, USA) in 20% methanol/10% acetic acid in distilled water, and then destained by rinsing with 20% methanol/10% acetic acid in distilled water. Active Hase was visualized as clear/white bands on the Alcian blue stained undigested HA background and imaged using an imaging densitometer (BioRad, GS 700).

4.2.12 Statistical analysis

Quantitative data for volumetric swelling ratio and elastic modulus were compared by ANOVA using Tukey's method for post-hoc comparisons. DNA content at 1 and 14 day time points were compared by two-tailed, unpaired t-tests. P values <0.05 were considered to be statistically significant.

4.3 Results

4.3.1 Synthesis of PEG-based macromers

4.3.1.1 Synthesis of PEG-bis-CP

PEG-bis-CP was synthesized by reaction of PEG and 2-chloropropionyl chloride in the presence of TEA in dichloromethane and the structure was characterized by ¹H-NMR using CDCl₃ as solvent (δ=3.4~3.90 (m, PEG backbone -CH₂), δ=4.3 (t, 2H, PEG terminal CH₂), δ=4.45 (t, 1H, 2-CP -CH), δ=1.7 (d, 3H, 2-CP -CH₃)). The degree of

substitution of the 2-CP to the terminal alcohol groups of PEG was calculated as 97% from the ratio of the integrals of the PEG back bone ($\delta=3.45\sim3.90$) and the terminal PEG methylene peaks ($\delta=4.3$)

4.3.1.2 Synthesis of PEG-bis-AP

PEG-bis-AP was synthesized by reaction of PEG-bis-CP and sodium acrylate in anhydrous DMF and the structure was characterized by ^1H -NMR using CDCl_3 as solvent (Figure 4.3, $\delta=3.45\sim3.90$ (m, PEG backbone $-\text{CH}_2$), $\delta=4.3$ (t, 2H, PEG terminal CH_2), $\delta=4.45$ (t, 1H, 2-CP $-\text{CH}$), $\delta=1.7$ (d, 3H, 2-CP $-\text{CH}_3$), $\delta=5.9$ and 6.4 (d, 2H, acrylic $-\text{CH}_2$), $\delta=6.15$ (q, 1H, acrylic $-\text{CH}$)). The degree of acrylation of the PEG-bis-AP was calculated as 87% from the ratio of the integrals of the PEG back bone ($\delta=3.45\sim3.90$) and the acrylate peaks ($\delta=4.3$).

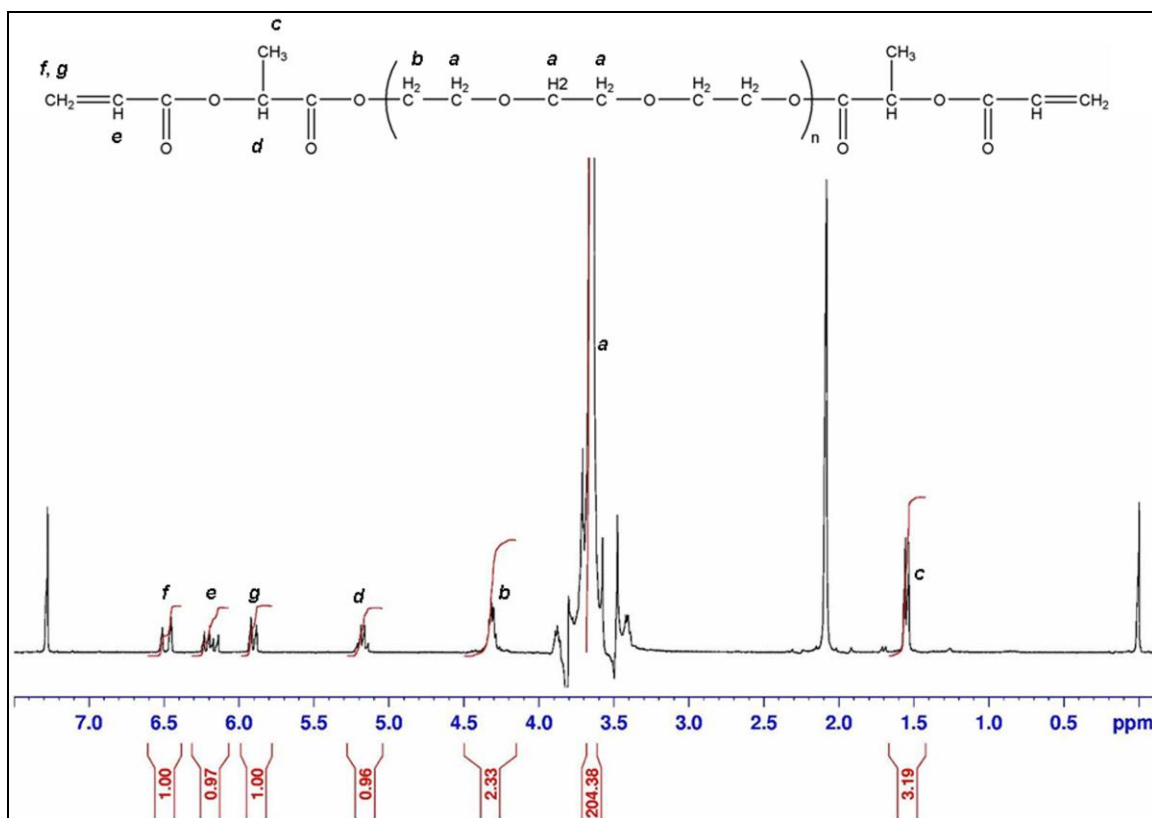


Figure 4.3 Structure and ^1H -NMR spectrum of PEG-bis-AP macromer.

4.3.2 Fibroblast spreading and proliferation in PEG diacrylate-based semi-IPNs

NDHF were photopolymerized within PEG-bis-AP hydrogels and semi-IPNs containing immobilized RGD cell adhesion peptides and enzymatically degradable ECM components. Fibroblasts in hydrogel controls exhibited a rounded morphology at 7 days (Figure 4.4A) and throughout extended periods in culture. Fibroblasts cultured in semi-IPNs containing collagen (Figure 4.4B) and gelatin (4.4C) retained a rounded morphology, similar to the hydrogel controls. Incorporation of HA resulted in fibroblast spreading and the development of a polarized morphology with multiple thin processes (Figure 4.4D).

Based on this observation we performed a dose-response experiment to determine the effect of varying HA content on fibroblast spreading. Confocal microscopy was performed to ensure that fibroblast spreading was occurring within the three-dimensional bulk of the materials as opposed to at the surface. Semi-IPNs containing 0.12% (Figure 4.5C) and 0.18% HA (Figure 4.5D) supported fibroblast spreading, while those containing 0.06% HA (Figure 4.5B) were comparable to control hydrogels (Figure 4.5A).

Fibroblasts exhibited spread, polarized morphology throughout the three-dimensional gel as shown in figure 4.6, which is a representative composite figure of confocal image sections taken at 100 μm intervals from 0 to 500 μm and pseudo-colored as a function of depth.

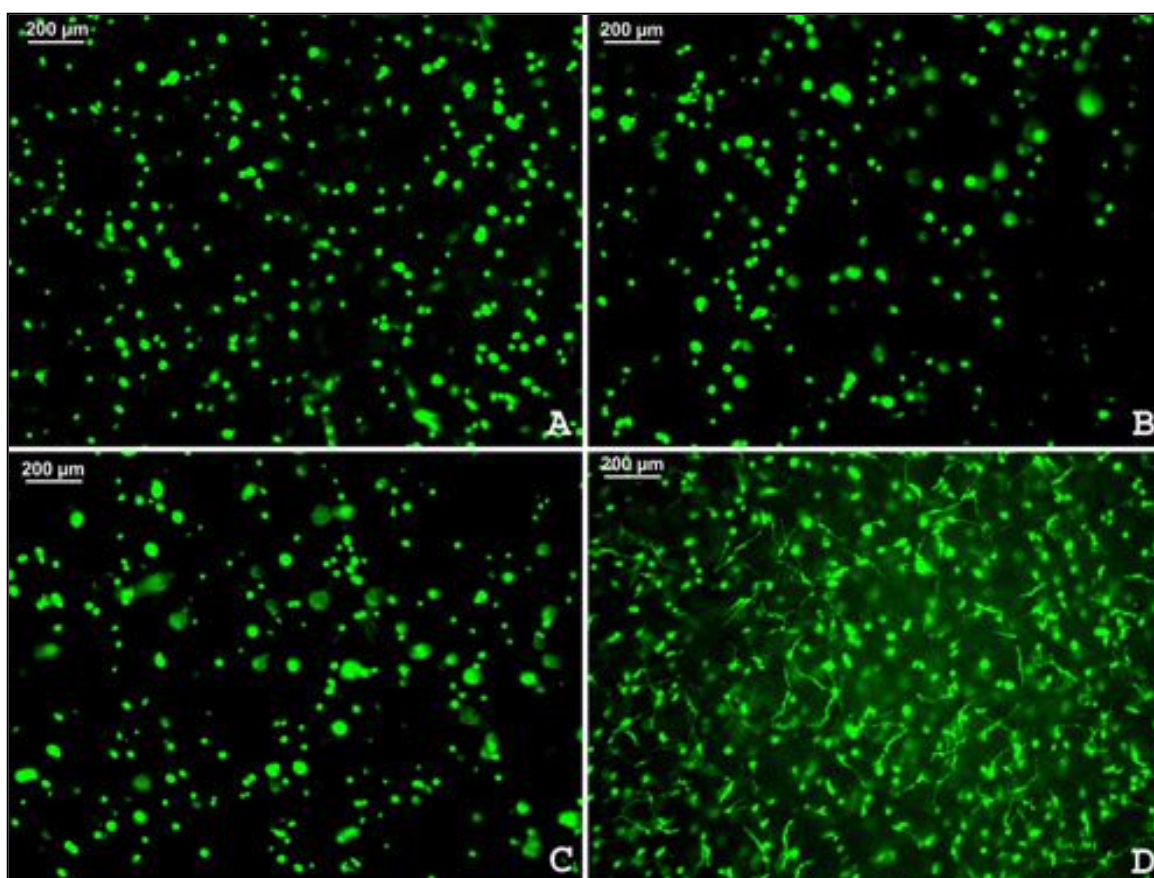


Figure 4.4 Viability and morphology of fibroblasts encapsulated in 6% w/v PEG-bis-AP hydrogels (A) and semi-IPNs containing 0.12 % w/v gelatin (B), collagen (C) and hyaluronic acid (D) visualized by staining with fluorescein diacetate after 7 days in culture. All scale bars are 200μm.

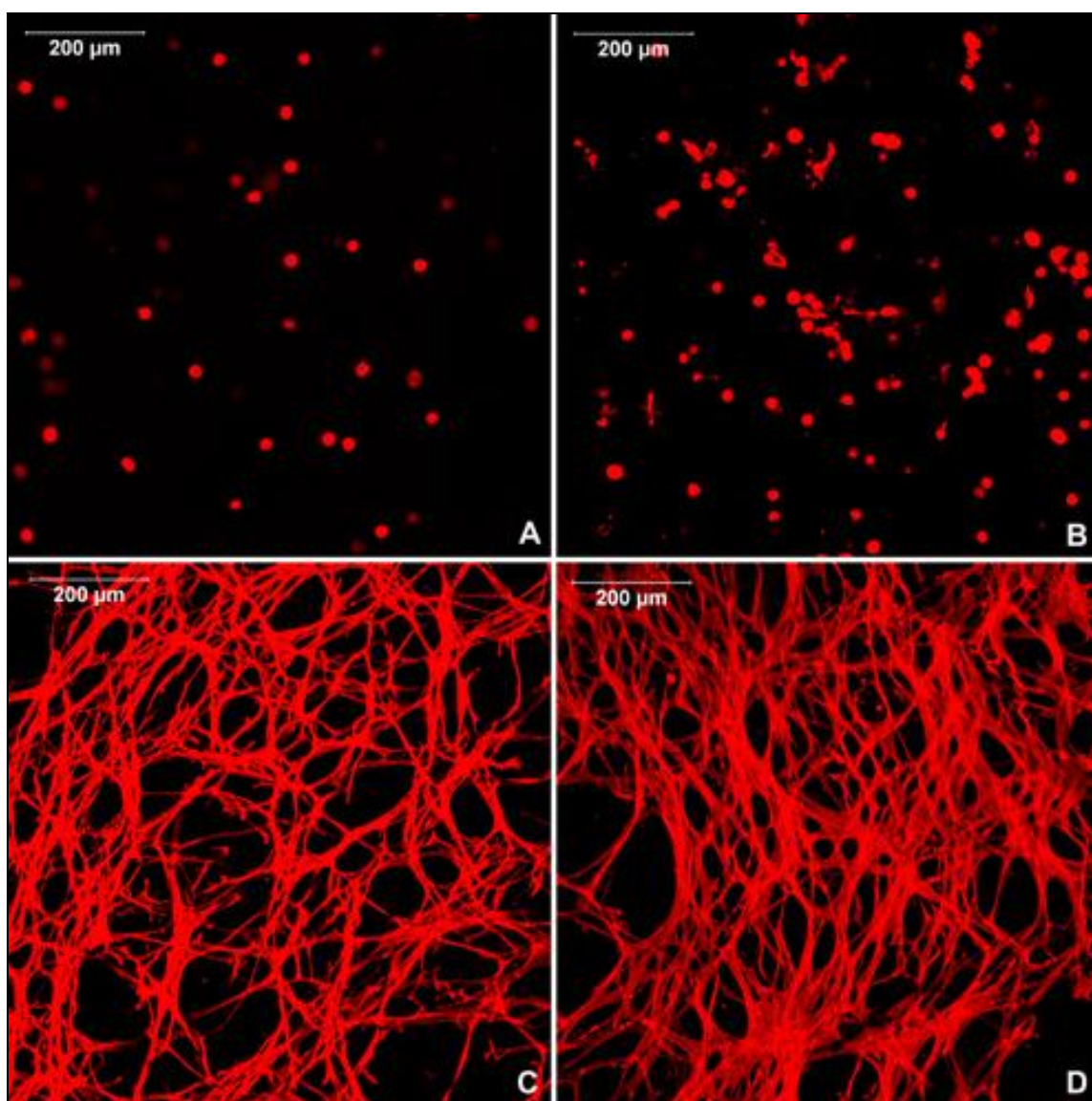


Figure 4.5 Confocal analysis (200 μm depth) of fibroblast morphology and spreading within 6 % w/v PEG-bis-AP hydrogels (A) and semi-IPNs containing 0.06% w/v (B) 0.12% w/v (C) and 0.18% w/v (D) hyaluronic acid after 14 days in culture. All scale bars are 200 μm .

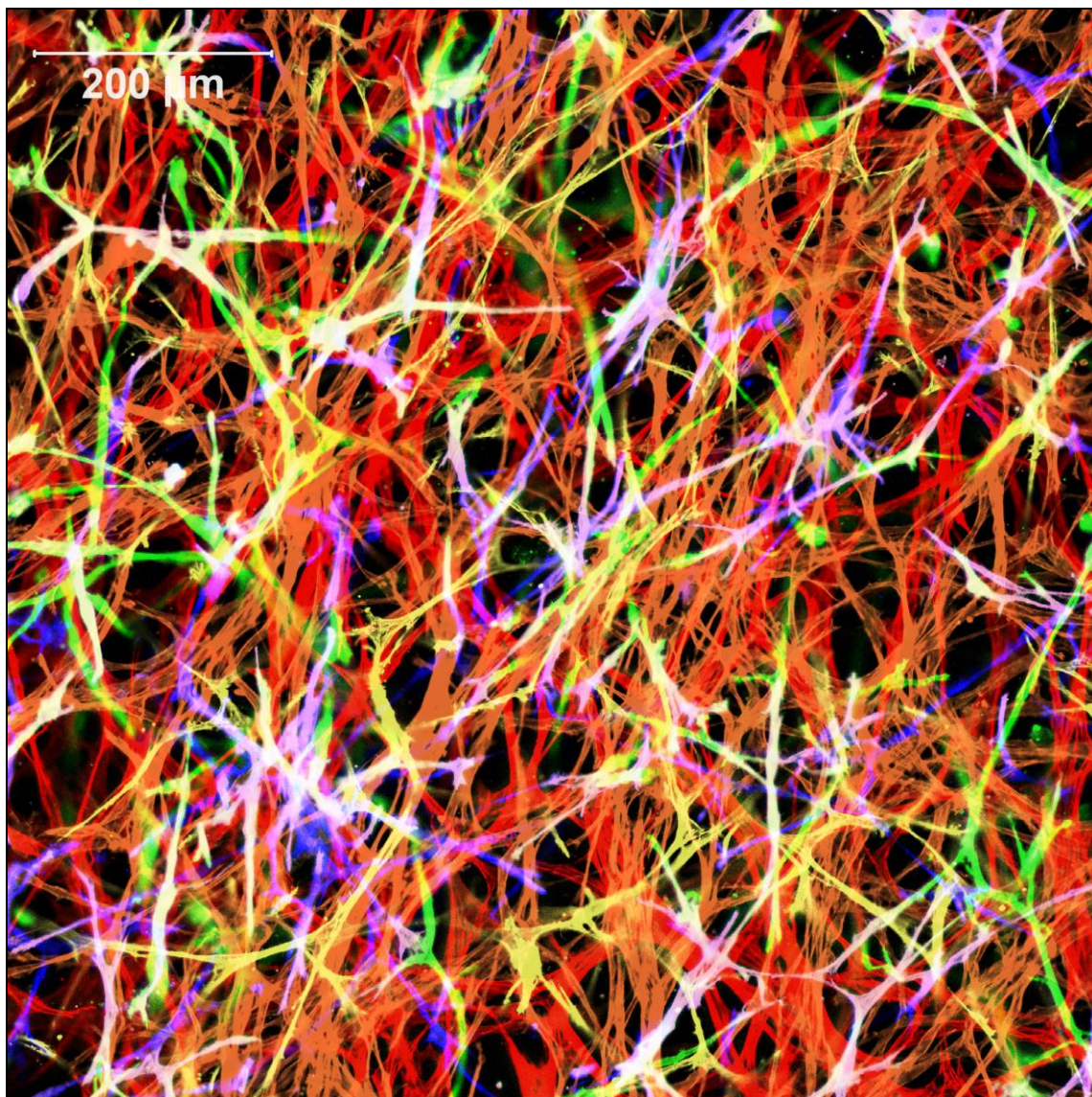


Figure 4.6 Representative pseudo-colored composite of confocal image sections of fibroblasts at varying depths within 6% w/v w/v PEG-bis-AP based semi-IPN containing 0.12% w/v hyaluronic acid after 14 days in culture. Color scheme – red (0 μm), orange (100 μm), yellow (200 μm), green (300 μm), pink (400 μm) and blue (500 μm). All scale bars are 200μm.

In order to determine whether the observed changes in fibroblast morphology corresponded with changes in cellular bioactivity, fibroblast proliferation was determined based on measurements of DNA content at 1 and 14 days. No significant difference was observed in the number of encapsulated fibroblasts at the day 1 time point among the varying gel formulations (Figure 4.7). After 14 days in culture, the number of fibroblasts significantly decreased in PEG-bis-AP hydrogels and semi-IPNs containing 0.06% HA. In contrast, a significant increase in cell number was observed for semi-IPNs containing 0.12% and 0.18% HA.

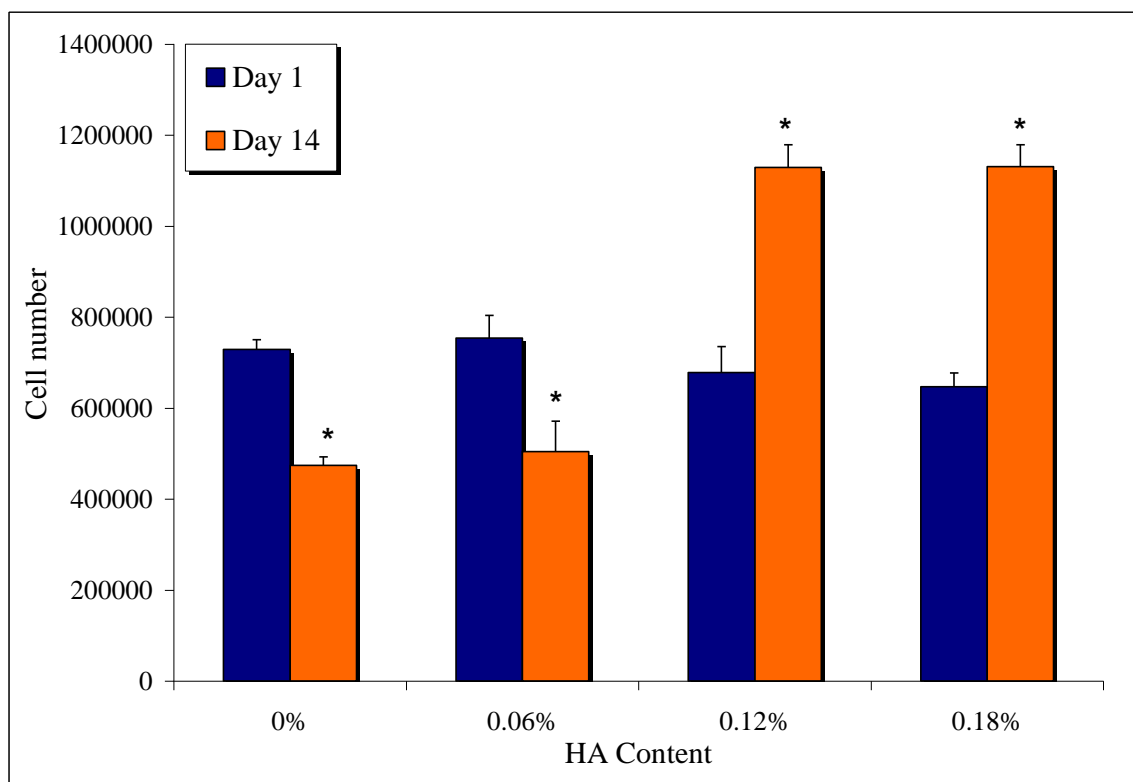


Figure 4.7 Fibroblast proliferation within 6% w/v PEG-bis-AP hydrogels and semi-IPNs containing varying amounts of HA.

4.3.3 Physical properties of PEG-bis-AP hydrogels and semi-IPNs containing HA

The effect of HA incorporation on hydrogel physical/chemical properties was assayed by measurements of swelling, tensile properties, and hydrolytic degradation. Incorporation of HA (0.06%, 0.12%, and 0.18%) significantly increased the volumetric swelling ratio of PEG-bis-AP/HA semi-IPNs relative to PEG-bis-AP hydrogels (Figure 4.8).

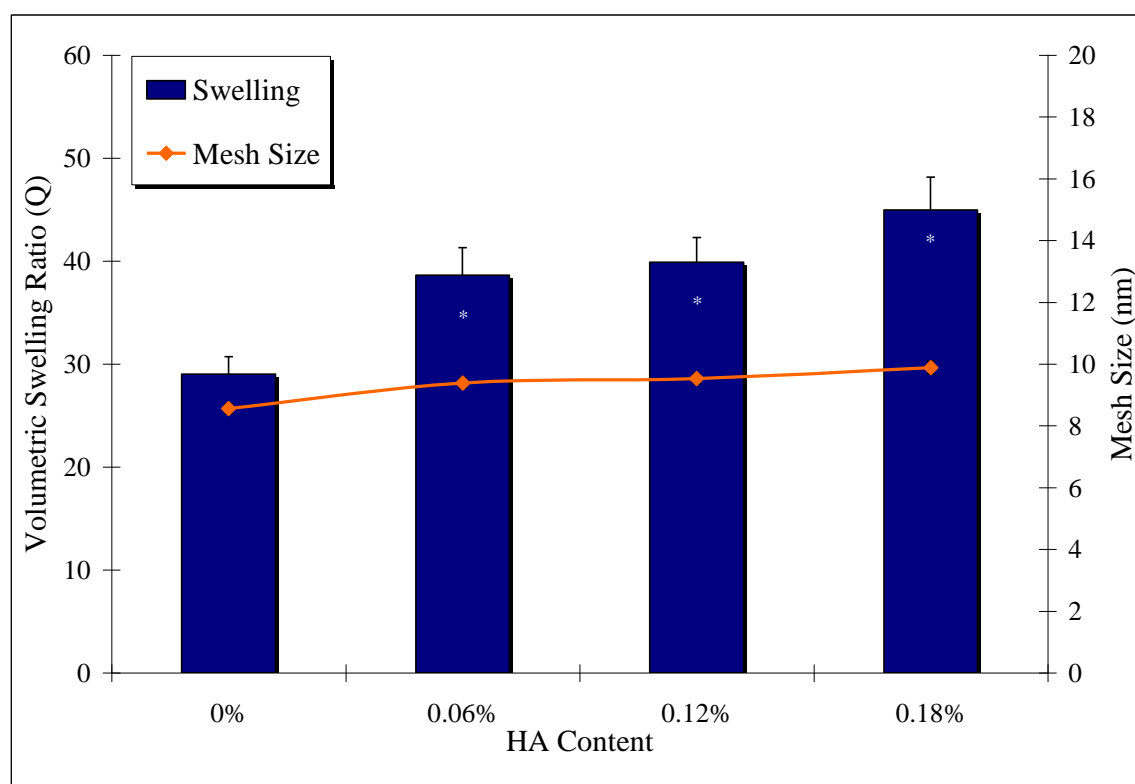


Figure 4.8 - Volumetric swelling ratio and mesh size of 6% w/v PEG-bis-AP hydrogels and semi-IPNs containing varying amounts of HA.

Similarly, PEG-bis-AP semi-IPNs containing 0.12% and 0.18% HA exhibited significantly increased elastic modulus relative to hydrogel controls (Figure 4.9).

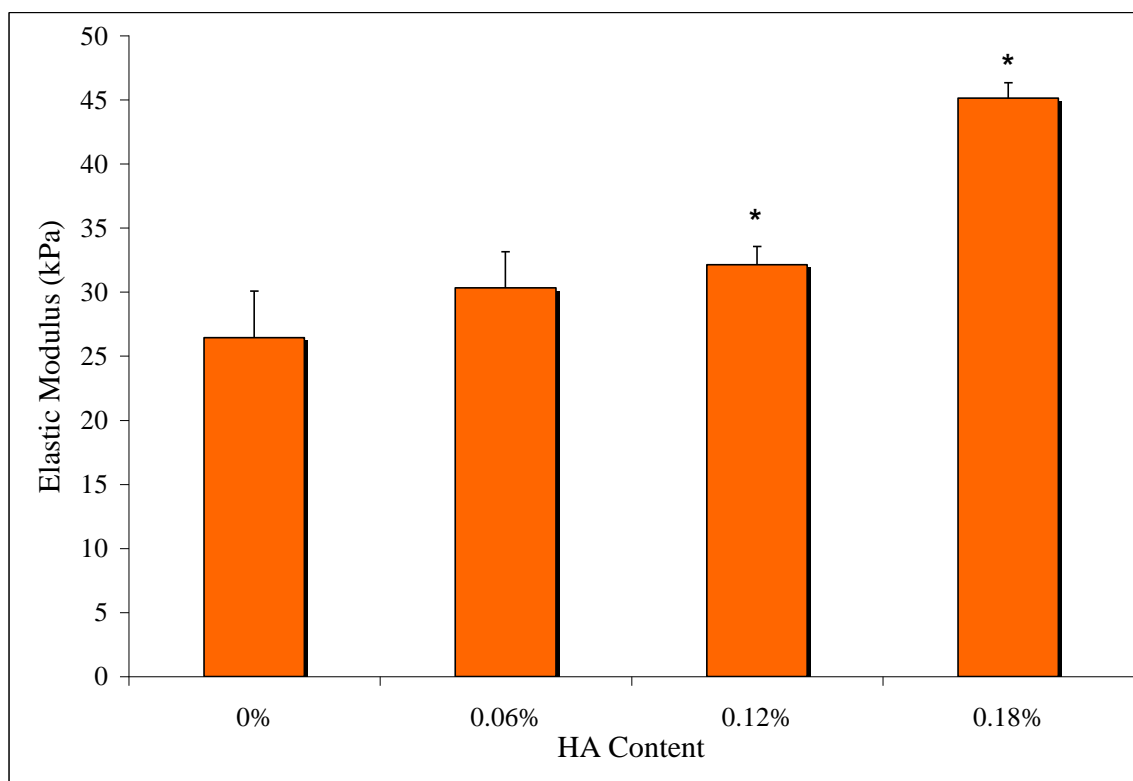


Figure 4.9 - Elastic modulus of 6% w/v PEG bis-AP hydrogels and semi-IPNs containing varying amounts of HA.

The hydrolytic degradation of PEG-bis-AP hydrogels and semi-IPNs containing varying amounts of HA was characterized on the basis of mass loss. The addition of HA did not significantly affect mass loss observed at varying time points during the study, although semi-IPNs containing 0.12% and 0.18% HA were completely degraded at 32

days versus 35 days for semi-IPNs containing 0.06% HA and control hydrogels (Figure 4.10).

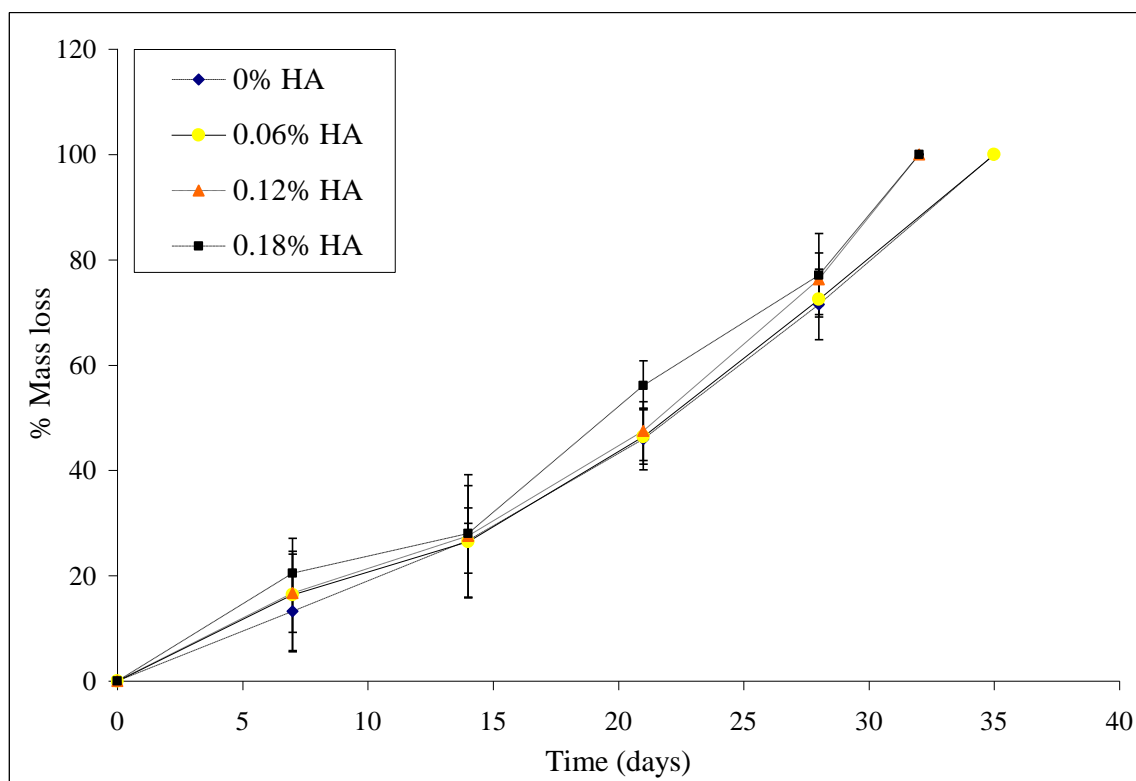


Figure 4.10 - Mass loss of 6 % w/v PEG-bis-AP hydrogels and semi-IPNs containing varying amounts of HA.

4.3.4 Contribution of HAse activity to fibroblast spreading in PEG-AP/HA semi-IPNs

In order to determine the contribution of HA enzymatic degradation to increased fibroblast spreading within HA-containing semi-IPNs, fibroblasts were encapsulated in PEG-bis-AP semi-IPNs containing 0.12% HA and cultured under routine conditions and

in the presence of two HAs inhibitors, neomycin trisulfate and L-ascorbic acid-6-palmitate. The presence of either inhibitor eliminated fibroblast spreading within HA-containing semi-IPNs (Figure 4.11). The addition of comparable concentrations of each inhibitor to cultures on fibroblasts in tissue culture wells did not alter cell viability, spreading, or time to confluence relative to untreated controls (data not shown). Reverse hyaluronan gel zymography confirmed substantial decreases in HAs activity of cell lysates obtained from cells cultured in the presence of both inhibitors relative to untreated controls (Figure 4.12).

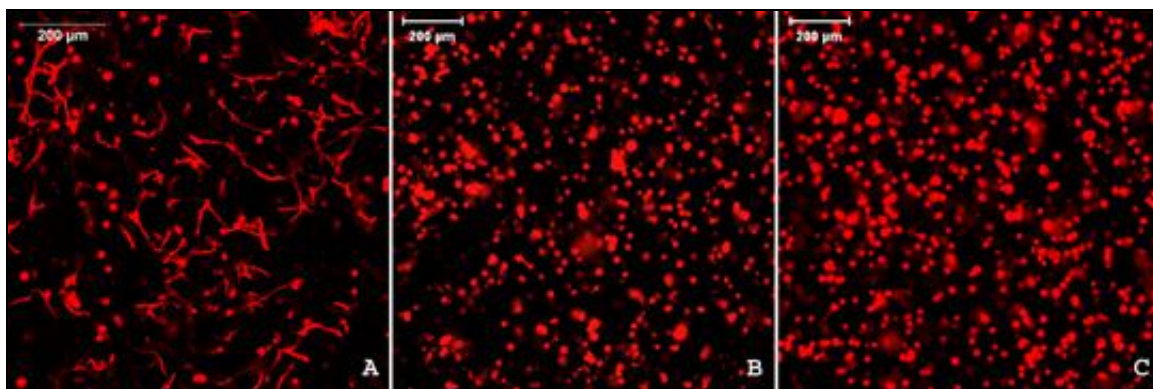


Figure 4.11 - Confocal analysis of fibroblast morphology and spreading within (200 μ m depth) 6 % w/v PEG-bis-AP based semi-IPN with 0.12 % w/v hyaluronic acid after 48 hours culture in normal media (A) and media containing 0.1mM Neomycin sulfate (B) and 0.1mM L-Ascorbic acid 6-palmitate (C).

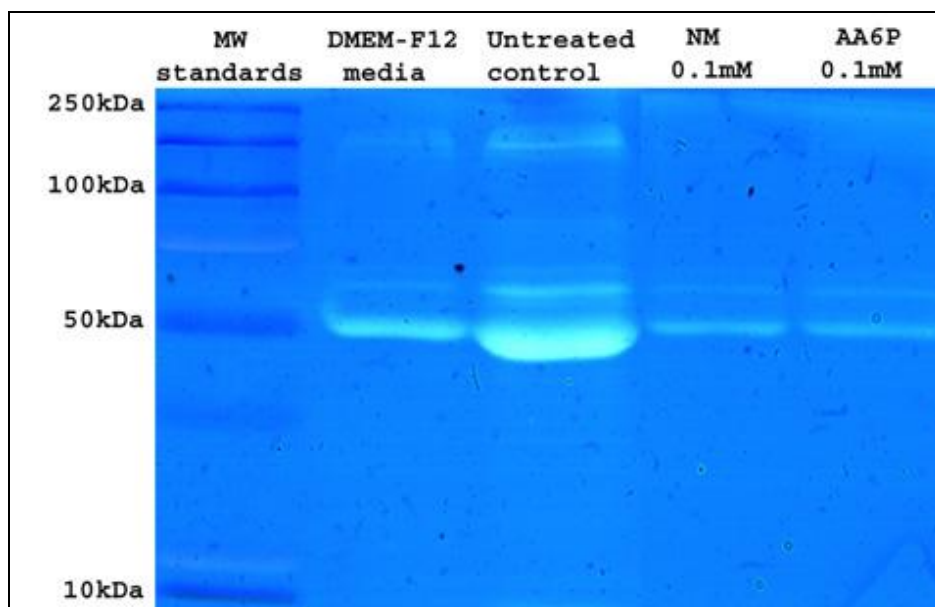


Figure 4.12 - Reverse HA gel zymography of extracts derived from fibroblasts encapsulated within 6% w/v PEG-bis-AP based semi-IPN containing 0.12% hyaluronic acid cultured with and without the presence of hyaluronidase inhibitors. Lane 1- molecular weight standard, lane 2-routine culture media, lane 3-extracts of fibroblasts cultured in routine media, lane 4-extracts of fibroblasts cultured in media containing 0.1mM Neomycin sulfate, lane 5-extracts of fibroblasts cultured in media containing 0.1mM L-Ascorbic acid 6-palmitate.

4.4 Discussion

Fibroblasts (~10 μ m diameter) encapsulated within hydrolytically degradable PEG-bis-AP hydrogels with nanometer-scale mesh size maintained a rounded morphology for up to 4 weeks of in vitro culture, consistent with results reported by others [224]. Cell shape is strongly correlated with biochemical function. Specifically,

rounded cell morphology is associated with limited proliferation and migration [255], key cellular processes in the early stages of tissue formation. In an attempt to introduce cell mediated degradability and enhance cell proliferation and migration, we have developed semi-IPNs based on synthetic, hydrolytically degradable PEG-bis-AP macromers and unmodified, enzymatically degradable ECM components such as collagen, gelatin, and HA. All materials were selected based on their well characterized biocompatibility. Hydrolytic degradation of ester bonds within the PEG network is expected to yield polyacrylic acid, lactic acid, and PEG. The lactic acid will be metabolized by the body, whereas the polyacrylic acid and the PEG will be eliminated by renal clearance [256]. Enzymatic degradation of collagen, gelatin, and HA produces simple amino acids and sugars that can be recycled in cellular metabolic pathways.

Among the three ECM components tested, PEG-bis-AP semi-IPNs containing HA supported extensive fibroblast spreading with the development of a multi-polar morphology within 48 hours. PEG-bis-AP semi-IPNs containing HA also supported significant increases in cell number, in contrast to control PEG-bis-AP hydrogels which exhibited significant decreases in cell number. Although fibroblasts are not as sensitive to apoptosis as epithelial or endothelial cells, these results may indicate that prolonged restriction to a rounded morphology is detrimental to their survival. Collagen- and gelatin-containing semi-IPNs did not support fibroblast spreading even at 2 and 4 week time points (data not shown). Thus, incorporation of HA uniquely supported a transition from a rounded to spread morphology and corresponding proliferative phenotype needed in the early stages of regeneration of many tissues. Several previous studies have

examined the effects of addition of native ECM material to synthetic hydrogels. Incorporation of collagen within a crosslinked network formed from acrylated Tetronic copolymers improved cell adhesion and spreading on the surface of the gels [244]. However, encapsulated hepatocytes maintained a rounded morphology [243]. The extensive remodeling observed in PEGylated fibrinogen networks [224] may result from much higher concentrations of protein and its covalent incorporation into the network. In the present studies, the concentration of ECM components in the semi-IPNs was kept low in order to minimize effects on the physical properties of the networks.

Incorporation of HA may increase cellular activity through several potential mechanisms affecting both the structure of the crosslinked network and cellular biochemical processes. Since fibroblast dimensions are several orders of magnitude greater than the typical mesh size of PEG diacrylate networks, the mesh size must increase either locally or throughout the material to facilitate cell spreading. Hydrogel porosity can be increased through the incorporation of leachable compounds, and it is plausible that HA functions in this manner. Increased cell density was observed at the sample edges after 2 weeks in culture (data not shown). This may result from localized leaching near the sample edges or from incomplete polymerization due to oxygen inhibition at the sample edges which were in contact with the ambient atmosphere. However, confocal analysis confirmed that cell spreading occurred throughout the 1 mm sample thickness. Since diffusional release of macromolecules from crosslinked hydrogel networks is dependent on molecular weight and limited release of intermediate size proteins such as IgG (150kDa) has been reported [257], it is unlikely that leaching of high

molecular weight HA significantly contributed to the increased cell spreading observed throughout the bulk of the semi-IPN materials.

Alternatively, HA could affect cellular activity by changing the network physical/mechanical properties. Although significantly increased swelling was observed in all HA-containing semi-IPNs, the corresponding change in mesh size is insufficient to affect fibroblast spreading. Similarly, the increases observed in semi-IPN mechanical properties were relatively small compared to order of magnitude changes previously shown to affect cell spreading and proliferation [258, 259]. Incorporation of HA also did not significantly affect hydrolytic degradation rate at early time points. Another potential mechanism is localized increases in mesh size resulting from HA degradation. HA can be degraded by both enzymatic and non enzymatic mechanisms [260]. Many studies have reported free radical-mediated depolymerization of HA [261, 262], which could also occur as a result of the free radical crosslinking method used in these studies. Enzymatic degradation of HA is mediated by the family of Hase enzymes. Although the precise mechanisms of HA degradation are still under investigation, the current model is based on extracellular binding of HA by membrane-bound hyal-2, which cleaves high molecular weight HA into approximately 20kDa fragments [263, 264]. Following internalization by CD44 or receptor for HA mediated motility (RHAMM), HA is further degraded into small oligosaccharides by hyal-1 and -2 in the lysosomal compartment at acidic pH (3.7 – 4.0) [265]. The contribution of enzymatic HA degradation was investigated by culturing fibroblasts in HA-containing semi-IPNs in the presence of two Hase inhibitors, neomycin trisulfate hydrate (0.1mM) [251] and L-ascorbic acid 6-

palmitate (0.1mM) [250]. Substantial reductions in Hase activity at approximately 50kDa were detected by reverse HA gel zymography developed at lysosomal pH as previously described [266]. The absence of Hase activity at pH 7.6 is consistent with the reported optimal activity of hyal-1 and -2 at lysosomal pH, however, hyal-2 activity at physiological pH has been reported using highly sensitive fluorescent substrates [267]. Hase inhibition was accompanied by the complete elimination of fibroblast spreading, demonstrating that enzymatic degradation of HA is a necessary mechanism of fibroblast spreading in HA-containing semi-IPNs.

Exposure to high molecular weight HA and its degradation products may also have multiple effects on cellular biochemical activity that vary as a function of HA molecular weight, concentration and cell phenotype [268]. HA binding through the cell surface receptors CD44, RHAMM, and ICAM-1 plays a vital role in the regulation of morphogenesis, wound healing, metastasis, and inflammation [265, 269, 270]. However, PEG/HA semi-IPNs without RGD peptides did not support fibroblast spreading (data not shown), indicating that HA binding contributes little to cell spreading and additional, exogenous adhesive ligands are required. An HA-rich pericellular sheath is vital for vascular smooth muscle cell proliferation and migration [271]. At high concentrations, high molecular weight HA has been shown to inhibit cell proliferation [268], while low concentrations of oligomeric HA stimulate proliferation [260]. HA also has been shown to stimulate the migration of epithelial cells and cardiac fibroblasts [272, 273], while inhibiting endothelial migration [274]. Based on these studies, high molecular weight

and degraded HA may also affect fibroblast proliferation and spreading, however, these effects are secondary to the facilitation of enzymatic degradation.

4.5 Conclusion

In conclusion, these studies demonstrate that incorporation of HA to form semi-IPNs with synthetic, hydrolytically degradable PEG diacrylate macromers improves fibroblast spreading and proliferation, and that cell-mediated, enzymatic HA degradation is the critical underlying mechanism. Addition of low levels of HA achieves substantial changes in cellular activity, while causing only relatively small changes in the network physical properties. This approach to accelerating the early stages of cellular remodeling may be useful for a variety of hydrogel-based tissue engineering approaches for vocal fold, vascular, and soft tissue applications. In addition, the angiogenic activity of low molecular weight HA degradation products released from the gels may also enhance their in vivo efficacy relative to conventional hydrogels [275].

CHAPTER 5

MECHANO-MIMETIC HYDROGELS FOR VOCAL FOLD LAMINA PROPRIA REGENERATION

5.1 Introduction

Voice is produced through the conversion of aerodynamic energy from exhalation to acoustic energy for sound production by the vibrating vocal folds and the upper respiratory tract. Voice is a primary “tool of trade” for teachers and sales personnel who comprise 4.2 and 13% of the US workforce [16]. Dysphonia (voice and speech impairment) characterized by increased effort to sustain phonation, decreased vocal quality, and total voice loss affects approximately 23% of the voice-dependent US workforce [14]; and may jeopardize job security, employment opportunities, and social interaction [225, 226].

The quality of voice depends on the biomechanical properties of the vocal fold tissue which derive from its ECM organization and composition [6]. The human vocal folds are multi-layered tissues comprised of a matrix rich lamina propria anchored to the vocalis muscle, and covered by a squamous epithelium. The fibroblast is the primary ECM producing cell type in the vocal fold tissue and is responsible for maintaining tissue homeostasis and regulating injury response [2]. The squamous epithelium and the superficial lamina propria together form the *vocal mucosa* which is abundant in hyaluronic acid, and proteoglycans like fibromodulin, decorin and versican which regulate the water content of the tissue and impart viscous shear properties to it [3, 4].

The vocal mucosa vibrates at frequencies between 100 – 1000Hz, and amplitudes of ~1mm [5]. In terms of mechanical properties the human vocal mucosa is characterized as a viscoelastic solid [276]. The intermediate and deep layers of the lamina propria together form the *vocal ligament*. The vocal ligament ECM is comprised mainly of elastin fibers which impart flexibility to the tissue, and collagen fibers which impart resistance to tensile stresses [6, 7]. The human vocal ligament shows non-linear stress-strain properties, characterized by 20 – 40kPa elastic modulus in the low strain linear region (0 – 15% strain), and values increasing to 600kPa in the high strain region (40% strain) [63]. It has been previously reported that the human vocal ligament can withstand a maximum of 60% strain [63, 277]. However, the human vocal ligament operates at low strain levels (0 – 15%) during normal phonation [8].

Vocal fold damage can be brought about by a multitude of factors like chronic misuse of voice, surgical intubation, and surgical treatment of infectious laryngeal diseases, laryngeal cancer, and benign pathologies like vocal folds polyps, nodules and edema [13, 14]. Traumatic or surgical injury of the lamina propria elicits a wound healing response that is characterized by the replacement of native parenchymal matrix with fibrous scar tissue. This change in the ECM composition leads to alterations in the tissue biomechanics which subsequently leads to dysphonia.

Voice therapy combined with the administration of steroids is the first line of therapy when presented with vocal fold defects and scars [13, 278]. However, voice therapy is ineffective when presented with scars in combination with pathologies like vocal polyps, nodules and edema despite rigorous adherence to the prescribed habits [19].

Synthetic, non degradable materials like pHEMA hydrogels [23], Teflon [20-22], ePTFE [24, 25], calcium hydroxyapatite [26], dacron [27], polydimethylsiloxane [28, 31] have been injected into the vocalis muscle in an attempt to overcome scarring-induced glottic incompetence through vocal fold medialization. However, physiological limitations such as migration from the site of implantation, foreign body granuloma formation, and fibrous encapsulation posed by these materials, and the theoretical advantages of natural, degradable biomaterials injected into the lamina propria have led to the use of materials like collagen, fat, fascia and HA for vocal fold augmentation purposes. In human subjects, though evaluations after six months of collagen injections indicated improvements in vibrational amplitude of the mucosal wave and amelioration of glottic incompetence, no perceptual improvement (acoustic analyses) was recorded [32]. Autologous fat [33, 35, 36] and fascia [37] injections yielded positive results short term but reports of these studies are inconsistent in terms of evaluations of glottic closure, amplitude of the mucosal wave and the effect on the stiffness of scar tissue. Injectable crosslinked HA gels (Hylan B gels) have been shown to improve mucosal wave amplitudes and glottic closure for approximately 12 months [38-40]. Material resorption and need for re-injection were the disadvantages associated with the use of natural materials like collagen, fat, fascia, and HA [41]. Current regeneration strategies have investigated the use of crosslinked composites of polyethylene glycol diacrylate (PEGDA)/derivatized HA [42, 43], composite collagen hydrogels [44], and HA-based microgels [279] as scaffolds for cell transplantation and matrix formation, as well as the

administration of hepatocyte growth factor (HGF) [46-48] and basic fibroblast growth factor (bFGF) [49] to reduce scarring during natural healing.

Previous studies by Titze et al. [11] highlighted the importance of the vibratory epigenetic stimulus in the vocal fold tissue. In vitro bioreactor experiments demonstrated that fibroblasts cultured in 3D, macroporous, elastomeric scaffolds under vibratory conditions exhibited significant differences in ECM-related gene expression relative to static controls, including increases in HA synthase 2 and proteoglycan genes consistent with the biochemical composition of the human vocal mucosa. Our overall hypothesis is that rapid restoration of the vibratory microenvironment will minimize scarring and facilitate tissue-specific matrix production, ultimately leading to restoration of normal phonation. Towards this end, the objective of this study was to create hydrogel formulations matching the viscous and elastic mechanical properties of the vocal mucosa and vocal ligament, respectively. These hydrogels will serve as:- (1) a model for studying the effects of ECM and biomechanics on fibroblast biology (2) potential injectable scaffolds for lamina propria regeneration.

5.2 Materials & Methods

5.2.1 Materials

Polyethylene glycol (PEG, MW=3934) was purchased from Fluka (Seelze, Germany) and 2-chloropropionyl chloride, 4-chlorobutyl chloride, triethylamine, dichloromethane (HPLC grade), anhydrous sodium sulfate, and sodium acrylate were obtained from Aldrich (St. Louis, MO, USA). Dimethyl formamide (extra dry) and ethyl

ether were purchased from Acros (Morris Plains, NJ) and Fisher Chemical (Fair Lawn, NJ), respectively. 2-hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone (I-2959) was obtained from Ciba Specialty Chemicals (Basel, Switzerland). A 10% (w/v) stock solution of I-2959 was prepared in 70% ethanol. Adult normal human dermal fibroblasts (NHDF, CC-2511) obtained from a 39 year old Black woman were purchased from Biowhitaker (Rockland, ME, USA). DMEM F-12 50/50,1X with L-glutamine, 15mM HEPES/trypsin EDTA (0.05% trypsin/0.53mM EDTA in HBSS), and penicillin/streptomycin were obtained from Mediatech (Herdon, VA, USA). Bovine growth serum was purchased from Hyclone (Logan, UT, USA). Hyaluronic acid (HA, MW=1.4 x 10⁶) was purchased from Genzyme (Cambridge, MA, USA).

5.2.2 Synthesis of PEG-bis-(2-acryloyloxy propanoate) (PEG-bis-AP) and PEG-bis-(2-acryloyloxy butyrate) (PEG-bis-AB) macromers

PEG-diacrylate macromers (PEG-bis-AP, Figure 5.1A and PEG-bis-AB, Figure 5.1B) containing ester bonds with varying susceptibility to hydrolytic degradation were synthesized by a two-step reaction as previously described [245]. In the first step, an intermediate product was synthesized by activating the PEG hydroxyl groups with di-functional chemical intermediaries (2-chloropropionyl chloride or 4-chlorobutyryl chloride) to form ester linkages with variable alkyl spacers. PEG-bis-AB, and PEG-bis-AP were synthesized by adding terminal acrylate groups to the intermediate products by nucleophilic substitution between the terminal alkyl chloride groups and sodium acrylate. Final acrylation efficiencies of ~87 and ~94% for PEG-bis-AP and PEG-bis-AB,

respectively, were calculated from ^1H -NMR (Bruker 300 MHz, CDCl_3) spectra based on the ratio of peak integrals derived from acrylate and PEG backbone protons.

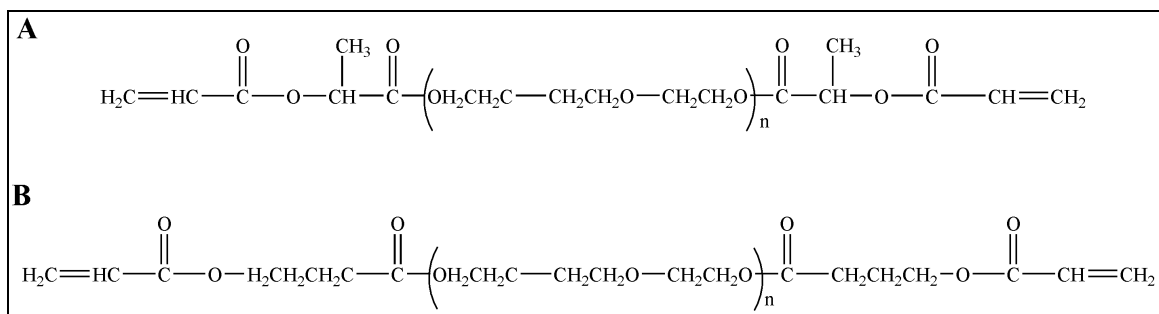


Figure 5.1 Chemical structures of PEG-bis-AP (A) and PEG-bis-AB (B)

5.2.3 Synthesis of methacrylated HA (GMHA)

GMHA (Figure 5.2) was synthesized by reacting sodium hyaluronate solubilized in double distilled water with a 20-fold molar excess of glycidyl methacrylate in the presence of triethylamine and tetrabutylammonium bromide as previously described [280]. The GMHA was precipitated in acetone, and dialyzed against double distilled water for 48 hrs. An ~7% degree of methacrylation was calculated from the ^1H -NMR (D_2O) spectra (Figure 5.3) based on the ratio of peak integrals derived from the methacrylate and HA carbohydrate protons, as previously described [281].

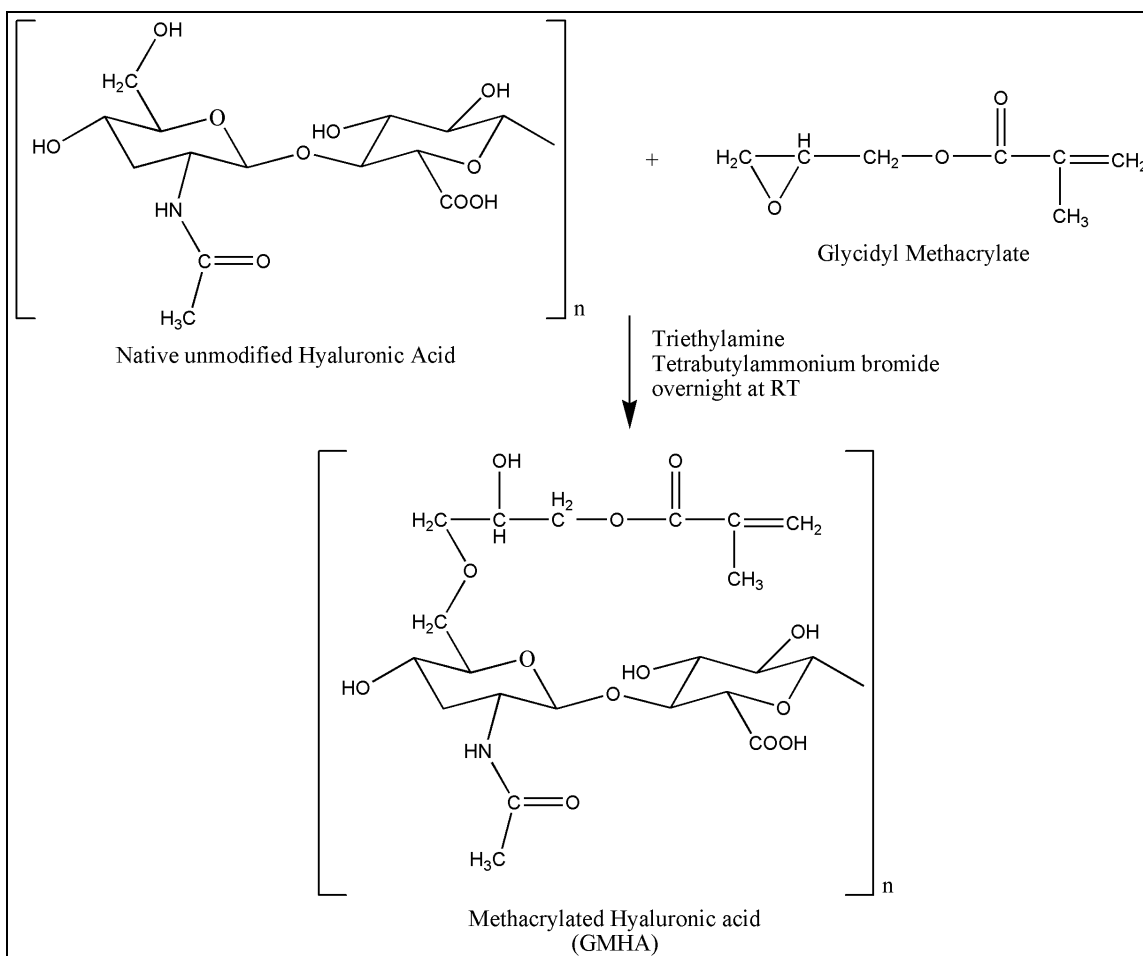


Figure 5.2 Chemical reaction scheme for GMHA macromers

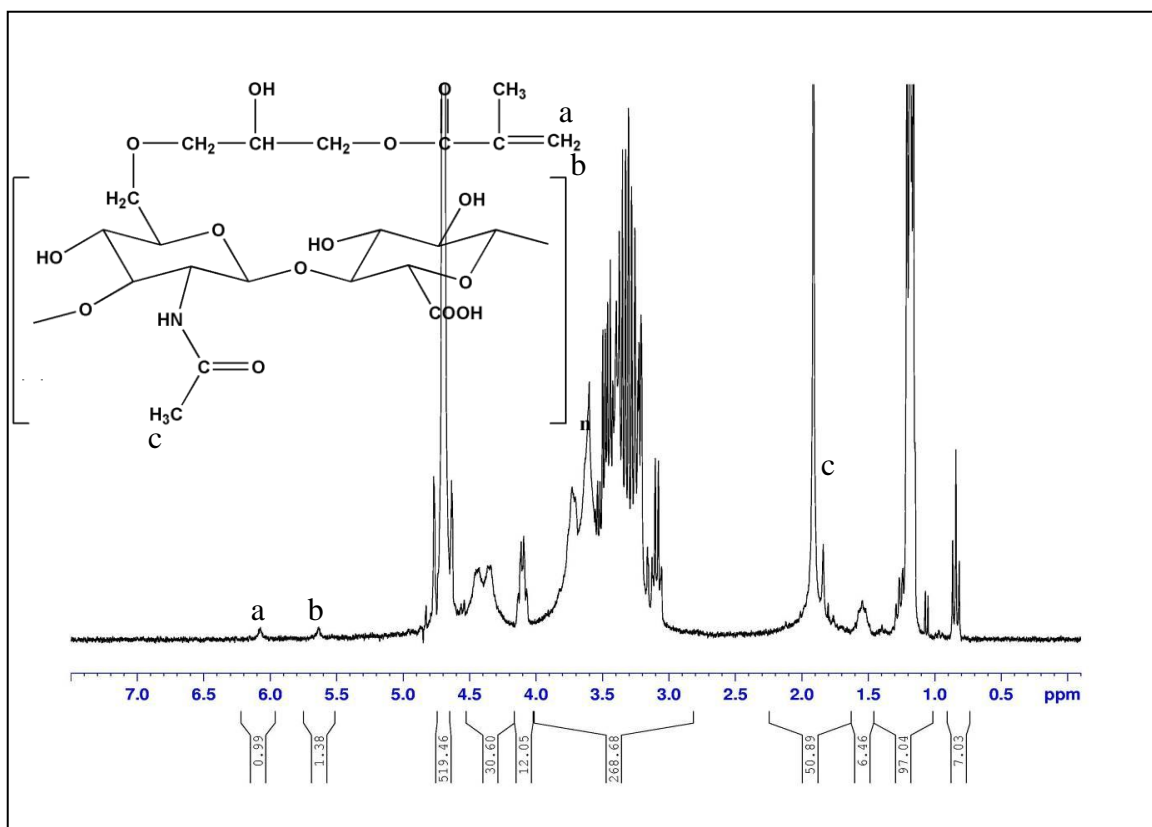


Figure 5.3 Structure and ^1H -NMR spectra of GMHA macromer

5.2.4 Cell culture

NHDF were routinely cultured in 182 cm² T-flasks using DMEM F-12 50/50,1X with L-glutamine and 15mM HEPES enriched with 10% v/v bovine growth serum and 50U/ml penicillin and 50μg/ml streptomycin. Culture medium was changed once every two days and cells were passaged weekly. All experiments were performed with cells from the 4th – 6th passages. For hydrogel encapsulation, monolayers of fibroblasts (ca. 95% confluence) were trypsinized, centrifuged, resuspended, counted in a hemacytometer, and adjusted to a final concentration of 1.6×10^7 cells/ml.

5.2.5 Hydrogel/Semi-IPN photopolymerization

Varying concentrations of PEG-bis-AP, PEG-bis-AB, PEG-bis-AB/AP (50:50 blend), and GMHA macromer solutions were prepared in 1X-PBS (0.1M, pH 7.4) and sterile filtered. During PEG-based semi-IPN synthesis, 0.12% w/v native unmodified HA was added to the PEG-bis-AP/AB macromer solution as explained previously [282]. A fibronectin-derived cell adhesion peptide, (GRGDS, Bachem, PA, USA) was conjugated to acrylate-PEG-NHS (MW=3400, Nektar) as previously described [216]. GRGDS-PEG-acrylate was prepared as a stock solution in 1X PBS (10 μ mol/ml) and sterile filtered. The macromer solutions with 0.1% w/v I-2959 initiator were photopolymerized between glass microscope slides separated by a 1 mm Teflon spacer by exposure to low intensity UV illumination (365nm, 10mW/cm², Black-Ray B100-AP, Upland, CA) for 7½ minutes (PEGDA hydrogels) and 6½ minutes (GMHA hydrogels) per side . For cell encapsulation studies, acrylate-PEG-GRGDS and NHDF were included at final concentrations of 2.5 μ mol/ml and 4 x 10⁶ cells/ml, respectively.

5.2.6 Mechanical characterization of photocrosslinked hydrogels

5.2.6.1 Elastic Modulus

Mechanical characterization of the PEG-based hydrogels was performed using hydrogels synthesized from the PEG-bis-AB macromer. PEG-bis-AB hydrogels (6, 8, and 10% w/v) were photopolymerized and cut into dumbbell shaped samples (gauge length 30mm, gauge width 5mm, and thickness 1mm). The samples were equilibrated in 1X-PBS overnight before testing. The samples (n = 4/group) were subjected to uniaxial

tensile testing using two different strain rates : (a) single cycle, 15% strain at 5mm/min and (b) multiple cycle (20 cycles), 15% strain at 1Hz using a 10N load cell in the MTS Synergie 100 (MTS Systems Corporation) at room temperature. Also, the 6% w/v PEG-bis-AB hydrogels were tested to failure at the 5mm/min strain rate. The sample ends were covered with small pieces of KimWipe to improve clamping stability, and each sample was tested twice to prevent errors due to sample slippage. Stress-strain curves were plotted, and elastic modulus was calculated as the slope of the of the curve stress-strain curve (0 – 15% strain). The PEG-bis-AB hydrogels were tested at room temperature since testing at 37°C would require the samples to be immersed in a buffer solution maintained at 37°C. In our experimental setup, during tensile testing the hydrogel samples were held in position using a Kim Wipe at both clamp ends to facilitate effective gripping (prevent slippage). Immersion of the test samples in any kind of liquid medium will result in significant inability to clamp the sample in place during the test. Hence the tensile tests were carried out at room temperature and not at 37°C. We believe that ~10°C increase in testing temperature will not have a significant effect on the elastic modulus of the material.

5.2.6.2 Dynamic viscosity & Elastic shear modulus

GMHA hydrogel samples were exposed to small amplitude oscillations over frequency sweeps from 0.01 to 15 Hz in conditions similar to those used to investigate the rheological properties of the human vocal mucosa [12]. Rheological characteristics as a function of polymer concentration (0.5, 1, 1.5, 2, 2.5 & 3% w/v) were investigated in a

stress controlled “ARES” Rheometer using parallel plate geometry. The hydrogel samples were placed in the gap (0.8mm) between the stationary upper plate and the rotating lower plate (diameter = 8mm), and the temperature was maintained at $37 \pm 0.1^{\circ}\text{C}$ by a ‘Peltier’ water cooling system. In order to determine the region of linearity, strain sweep tests were performed at fixed oscillation frequency (1Hz). Rheological tests were performed in the linear strain region (strain amplitude – 0.01rad), over a frequency range of 0.01 – 15Hz, covering 25 frequencies over 3 decades. The dynamic viscosities of the varying % w/v GMHA hydrogels were calculated by dividing the observed values of the loss moduli by the corresponding angular frequencies tested. The elastic shear moduli of the varying % w/v GMHA hydrogels were also recorded and plotted at all frequencies tested.

5.2.7 Degradation studies

PEG-bis-AP, PEG-bis-AB/AP_{50:50}, and PEG-bis-AB hydrogels (6% w/v) were photopolymerized, equilibrated with water for 24 hours to remove any unpolymerized macromer, lyophilized, and weighed (W_{d0}). Samples were incubated with 4ml of 1X-PBS containing 0.01% sodium azide (Sigma) in scintillation vials under shaking at 37°C . Buffer was changed once every two days. At weekly intervals, samples ($n = 3/\text{group/time point}$) were collected, washed with distilled water three times, lyophilized, and weighed (W_{dt}). Percent mass loss was calculated as $((W_{d0} - W_{dt})/W_{d0}) \times 100$. Mass loss (degradation) studies were not separately conducted for the GMHA hydrogels since the rate of degradation of these hydrogels depends on the amount of Hase produced by the

encapsulated cells. Also, the degradation of GMHA hydrogels in the presence of Hase has been previously reported by Leach et al [280].

5.2.8 Encapsulated fibroblast viability, morphology, and proliferation

NHDF were encapsulated in 6% w/v PEG-bis-AB/AP_{50:50} semi-IPNs containing 0.12% w/v HA [282] and 2% w/v GMHA hydrogels (n = 3 samples/experimental group). Samples were cultured in 12 well plates for 1-28 days.

5.2.8.1 Viability assessment

After 7 days in culture, hydrogel samples were stained with 1 μ M fluorescein diacetate (FDA, Molecular Probes, Eugene, OR) and 2.5 μ M propidium iodide (PI, Molecular Probes), rinsed with PBS, visualized and imaged by fluorescence microscopy (Zeiss Axiovert 200).

5.2.8.2 Confocal analysis of morphology

After 14 days in culture, samples from each group were fixed in 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100, and stained with Alexa 594-phalloidin (Molecular Probes). Samples were visualized and imaged using a Zeiss Confocal LSM510 microscope.

5.2.8.3 Fibroblast proliferation

Hydrogel/Semi-IPN samples from each group were collected at days 1, 7, 14 and 28, rinsed twice with sterile PBS, snap-frozen in an ethanol-dry ice bath, and stored at -80°C. DNA solubilization and quantitation were based on modifications of previously described methods [247]. Briefly, the samples were subjected to 3 freeze-thaw cycles in 1.4ml of 10mM EDTA, pH 12.3. Once the hydrogels were completely dissolved, the pH was neutralized by the addition of 100µl 1M potassium phosphate (monobasic) solution. Total DNA content was measured using the Picogreen DNA-binding dye (Molecular Probes) and a fluorescence microplate reader (Tecan GENios, excitation: 485nm, emission: 535nm). DNA content was converted to cell number based on a DNA standard curve prepared from serial dilutions of NHDF.

5.2.9 Encapsulated fibroblast ECM production

5.2.9.1 Sample preparation

NHDF were encapsulated in 6% w/v PEG-bis-AB/AP_{50:50} based semi-IPNs containing 0.12% w/v HA and 2% w/v GMHA hydrogels (n=6 samples/hydrogel type). Samples were cultured for 14 and 28 days (n=3 per time point), rinsed with sterile PBS, and fixed in 4% w/v paraformaldehyde. The fixed hydrogel samples were infiltrated, embedded and sectioned partly as explained elsewhere [283]. Briefly, the hydrogel samples were cryoprotected by rinsing with 5% sucrose in PBS for an hour followed by infiltration with increasing concentrations of sucrose in PBS (mixing 5% and 20% sucrose in PBS in the ratios 2:1, 1:1 and 1:2) for 3 hrs each. The cryoprotected samples

were then infiltrated with an ascending series of mixtures of sucrose in PBS and OCT (2:1, 1:1 and 1:2 ratios) for 6 hrs each at room temperature under constant vacuum. This step was followed by embedding in the OCT mold using 100% OCT solution. 8µm thick cryosections were cut and transferred to microscope slides for immunohistochemistry and toluidine blue O staining.

5.2.9.2 Immunohistochemistry

The cryosections were fixed in chilled acetone, incubated for an hour at room temperature with rabbit, α -human collagen type 1 IgG primary antibody (Chemicon, dilution 1:400), rinsed, and incubated 1 hour with secondary antibody (goat, anti-rabbit IgG, Alexa Fluor 488, dilution 1:220). Samples were coverslipped using Vectashield (Vector laboratories, Burlingame, CA) mounting medium containing DAPI for counterstaining cell nuclei.

5.2.9.3 Toluidine Blue O staining

Sulfated GAGs (s-GAGs) were stained by incubating the cryosections in an aqueous Toluidine Blue O solution (0.0714% toluidine blue O, 0.0714% pyronin Y, and 0.143% borax) for 6 minutes as previously described [44].

The slides were digitally imaged and analyzed using a Zeiss Axiovert 200 microscope.

5.2.10 Statistical analysis

All comparisons were made between binary data sets using two-tailed, unpaired t-tests and “p” values less than 0.05 were considered statistically significant.

5.3 Results

5.3.1 Mechanical Characterization of photocrosslinked hydrogels

Hydrogels/Semi-IPNs described here were aimed at restoring mechanical integrity and function for the vocal fold lamina propria. Due to the distinct biomechanical properties of the vocal mucosa and the vocal ligament, a two component system comprised of methacrylated HA (viscous, vocal mucosa) and PEG-diacrylate (elastic, vocal ligament) was developed. Variation of polymer concentration was investigated to identify formulations approximating the native tissue biomechanics.

5.3.1.1 Tensile testing

Uniaxial tensile tests showed that PEG-bis-AB hydrogel elastic modulus significantly increased with increasing macromer concentration (Figure 5.4). PEG-bis-AB hydrogels prepared at 6% w/v concentration were found to exhibit elastic modulus approximating the mean value ($33.1 \pm 10.4\text{kPa}$) reported for native human vocal ligaments at low strains (0 – 15%) [63].

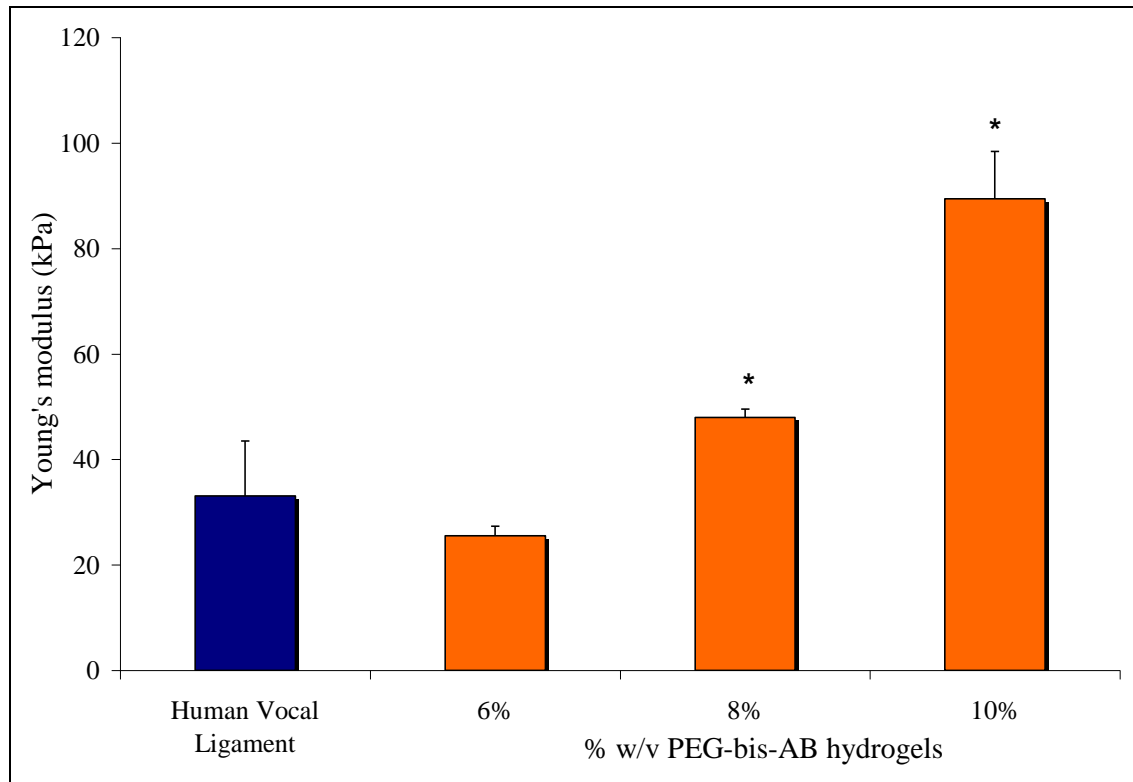


Figure 5.4 Elastic modulus of the human vocal ligament [63] and varying concentrations of the PEG bis-AB hydrogels. * indicates statistically significant differences relative to the human vocal ligament.

Figure 5.5 shows the typical stress–strain hysteresis cycles for tensile loading – unloading deformation of 6% w/v PEG-bis-AB hydrogels, tested to 15% strain at 1Hz. The linear stress-strain curve of the PEG-based hydrogels is consistent with previously reported stress-strain curves at low strain levels (0 – 15%) of the vocal fold ligament specimens tested [63, 103, 277, 284] and other biological soft tissues [285]. At higher strain levels the human vocal ligament indicated a non linear stress-strain response [63, 103, 277, 284]. Preconditioning is observed for excised biological tissues, with the

stress-strain hysteresis cycles shifting downward from the 1st cycle and stabilizing at the 15th – 20th cycle due to stress relaxation [63, 277] and re-alignment of elastic and collagen fibers following excision. However, preconditioning is not pronounced in the PEG-based synthetic hydrogels tested since stabilization of the loading-unloading curves occurs between the 1st and the 2nd cycles. The sharp reduction in stress levels at peak strain (15% strain) during the unloading curve of 1st cycle may be attributed to severing of weak crosslinks within the hydrogel mesh after which the material stabilized and exhibited repeatable loading-unloading curves between the 2nd and the 20th cycles. Linear curves with hysteresis, due to viscous energy loss were observed for all the samples tested. Similar viscoelastic behavior was observed for all the 6% w/v PEG-bis-AB hydrogels tested.

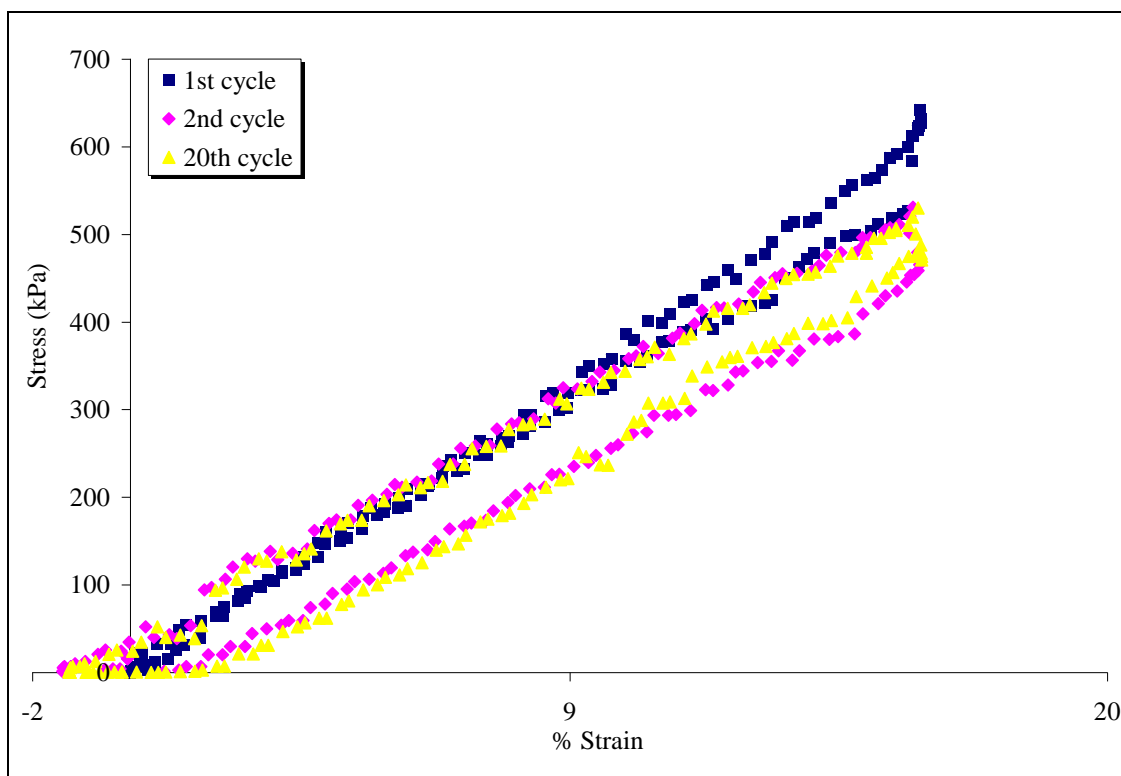


Figure 5.5 Typical loading – unloading cycles of the 6% w/v PEG-bis-AB hydrogels under tensile strain (strain rate = 1 Hz). The phenomenon of hysteresis is demonstrated, for the 2nd cycle and the 20th cycle tested.

The 6% w/v PEG-bis-AB hydrogels tested at 5 mm/min exhibited linear stress-strain characteristics until failure ($46 \pm 2\%$ strain) as shown in figure 5.6.

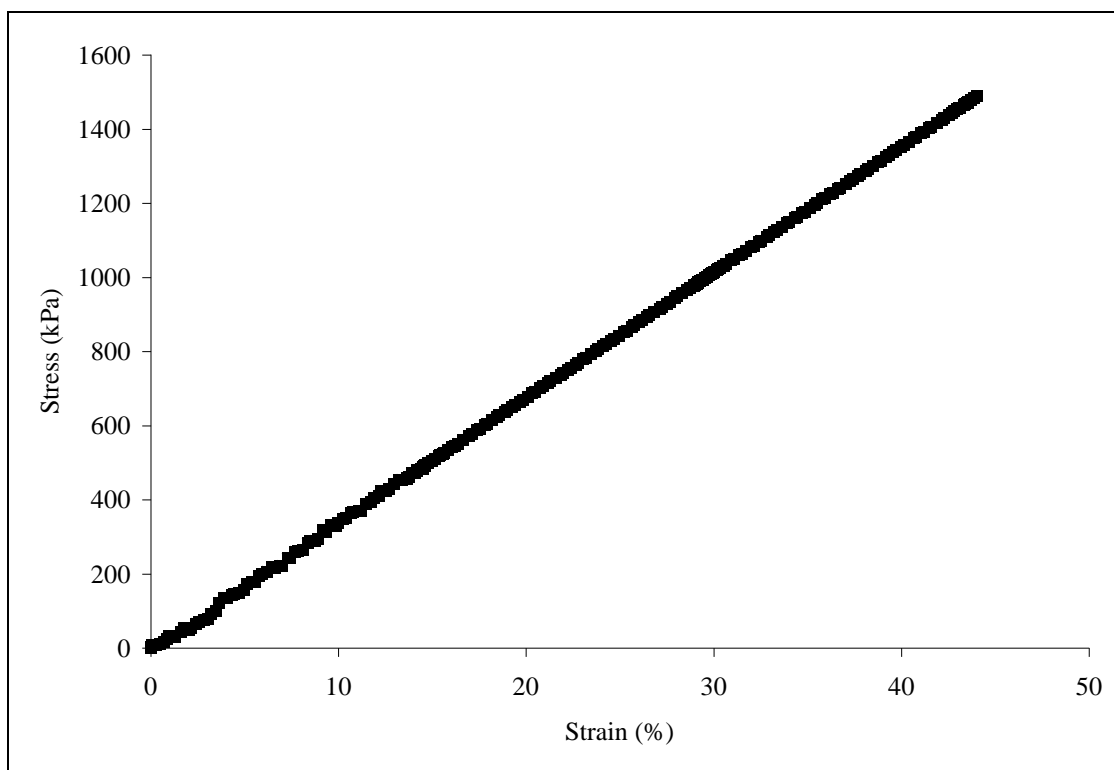


Figure 5.6 Typical stress-strain curve for 6% w/v PEG-bis-AB hydrogels strained until failure.

5.3.1.2 Rheological testing

Dynamic viscosities calculated over the range of the tested frequency sweep (0.01 – 15Hz) indicated that the 0.5 – 3% w/v concentrations of GMHA hydrogels demonstrated a shear thinning response similar to many soft tissues (Figure 5.7). Our results indicated that as the macromer concentration increased the magnitude of dynamic viscosity increased over the entire frequency range tested. GMHA gels prepared at concentrations ranging from 1.5 – 2.5 % w/v exhibited dynamic viscosities consistent with the range of properties reported for native human vocal mucosa.

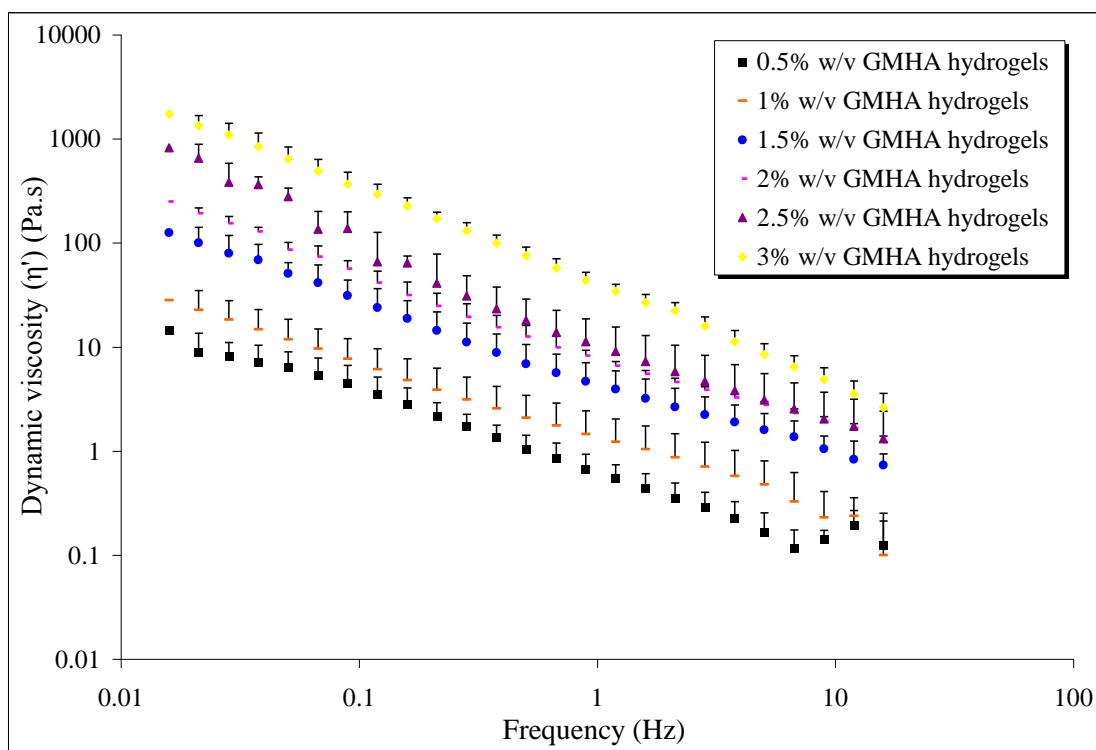


Figure 5.7 Dynamic viscosities of varying concentrations of GMHA hydrogels.

However, the 1.5 – 2.5% w/v GMHA hydrogels exhibited elastic shear modulus higher than that reported for the native human vocal mucosa (Figure 5.8).

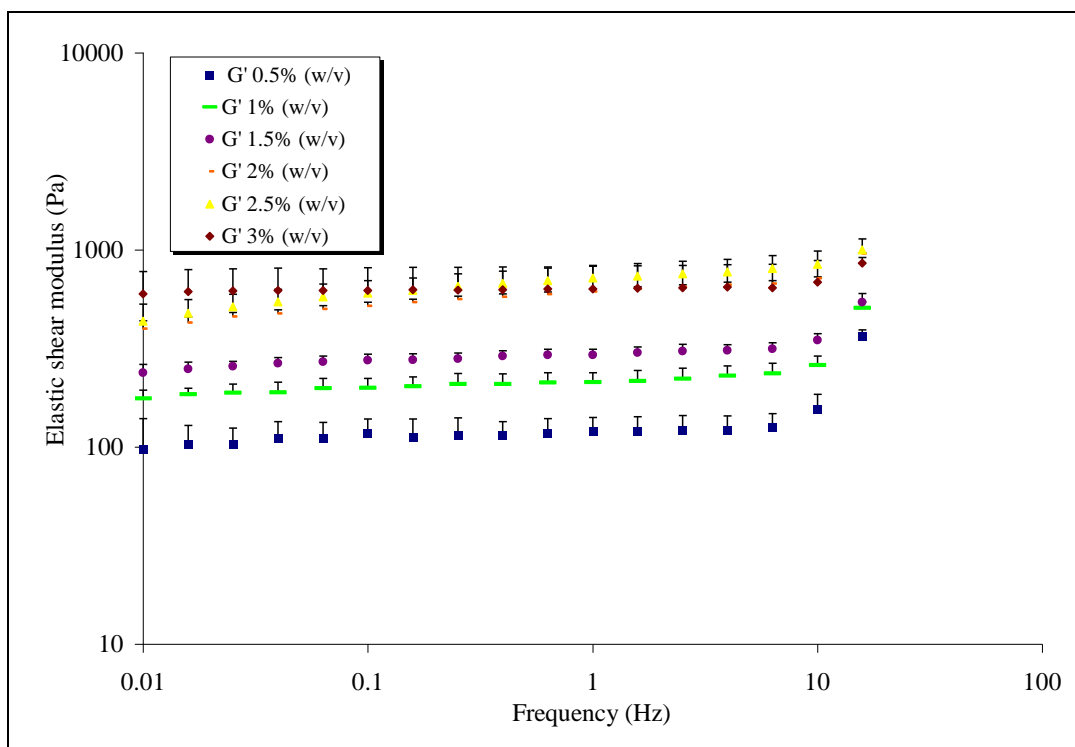


Figure 5.8 Elastic shear moduli of varying concentrations of GMHA hydrogels.

The dynamic viscosity of native HA, GMHA macromer, and crosslinked GMHA hydrogels was also tested at fixed 2% concentration. Uncrosslinked GMHA macromers exhibited an approximately order of magnitude decrease in dynamic viscosity relative to the native HA, while crosslinking substantially increased dynamic viscosity relative to both native HA and uncrosslinked GMHA (Figure 5.9).

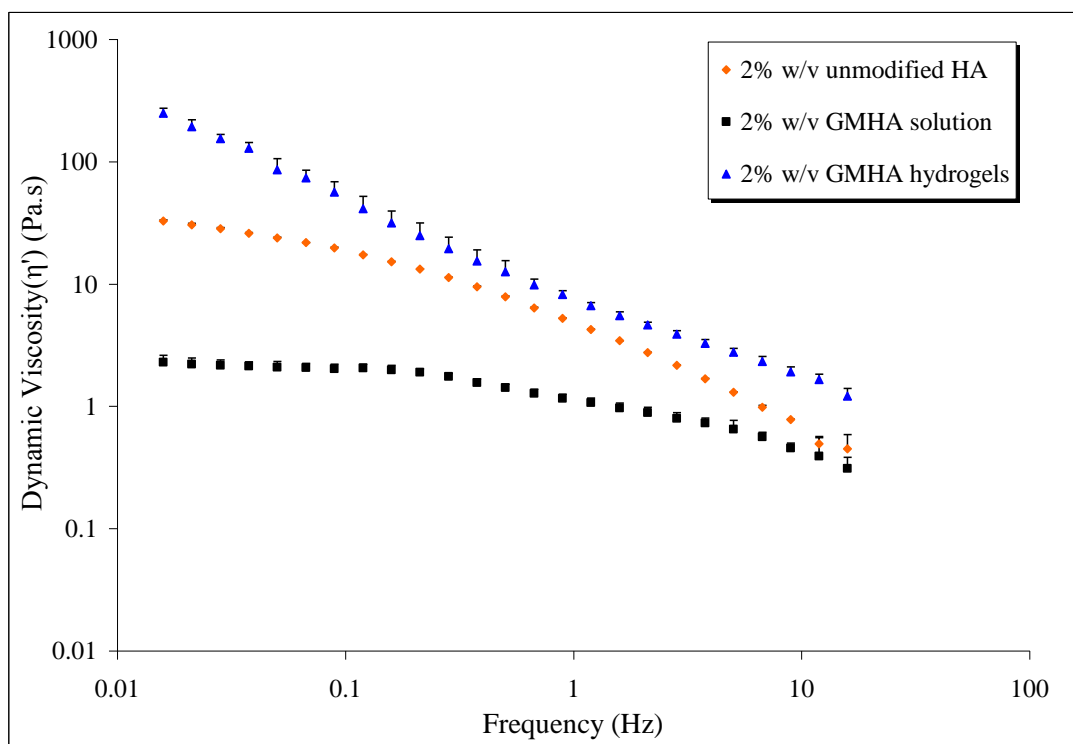


Figure 5.9 Dynamic viscosities of 2% w/v unmodified HA solution, GMHA solution and GMHA hydrogels.

5.3.2 Degradation studies

While the GMHA hydrogels are susceptible to enzymatic degradation, the PEG-diacrylate macromers were synthesized to contain ester bonds with varying susceptibility to hydrolytic degradation. The mass loss of three different photocrosslinked hydrogel formulations (PEG-bis-AP, PEG-bis-AB/AP_{50:50}, and PEG-bis-AB) at 6% w/v concentration was examined on a weekly basis. The 6% w/v PEG-bis-AP, PEG-bis-AB/AP_{50:50}, and PEG-bis-AB hydrogels degraded completely within 7, 11, and 18 weeks respectively (Figure 5.10).

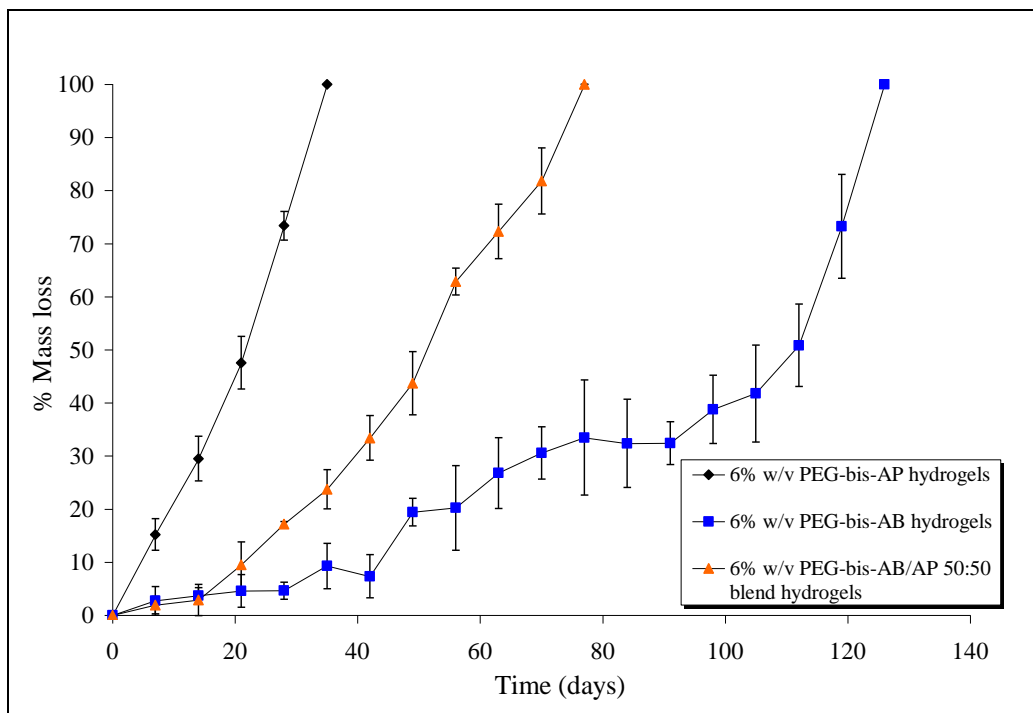


Figure 5.10 Mass loss of 6 % w/v PEG-bis-AP, PEG-bis-AP/AB_{50:50} blend and PEG-bis-AB hydrogels.

5.3.3 Fibroblast viability and proliferation in PEG-bis-AB/AP_{50:50} based semi-IPNs and GMHA hydrogels

NDHF were encapsulated within 2% w/v GMHA hydrogels, and 6% w/v PEG-bis-AB/AP_{50:50} blend semi-IPNs (containing 0.12% w/v HA) containing immobilized RGD cell adhesion peptides by photopolymerization. Fibroblasts encapsulated in the 6% w/v PEG-bis-AB/AP_{50:50} semi-IPNs exhibited spreading and the development of polarized morphology with multiple thin processes as shown in figure 5.11A. Fibroblasts encapsulated within the 2% w/v GMHA hydrogels exhibited spreading and a stellate

morphology as shown in figure 5.11B. High levels of viability were observed in all samples. Confocal microscopy was performed to ensure that fibroblast spreading was occurring within the three-dimensional bulk of the materials as opposed to at the surface. Fibroblasts exhibited a spread, polarized morphology throughout the volume of the PEG-bis-AP/AB_{50:50} blend semi-IPNs indicated by figure 5.11C which is a representative confocal image section taken at 200 μm within the semi-IPN. Cell spreading and cell-cell aggregation was detected throughout the bulk of the GMHA hydrogel as shown in figure 5.11D.

In order to determine whether the observed changes in fibroblast morphology corresponded with changes in cellular bioactivity, fibroblast proliferation was determined based on measurements of DNA content at 1, 7, 14, and 28 days. No significant difference was observed in the number of encapsulated fibroblasts at the day 1 time point among the PEG-bis-AB/AP_{50:50} semi-IPNs (0.12% w/v HA) and GMHA hydrogels as shown in figure 5.12. After 7, 14, and 28 days in culture, a significantly increased number of fibroblasts was observed in both PEG-based and GMHA gels relative to day 1. However, both the hydrogel types exhibited a significant decrease in cell number at day 28 relative to day 14.

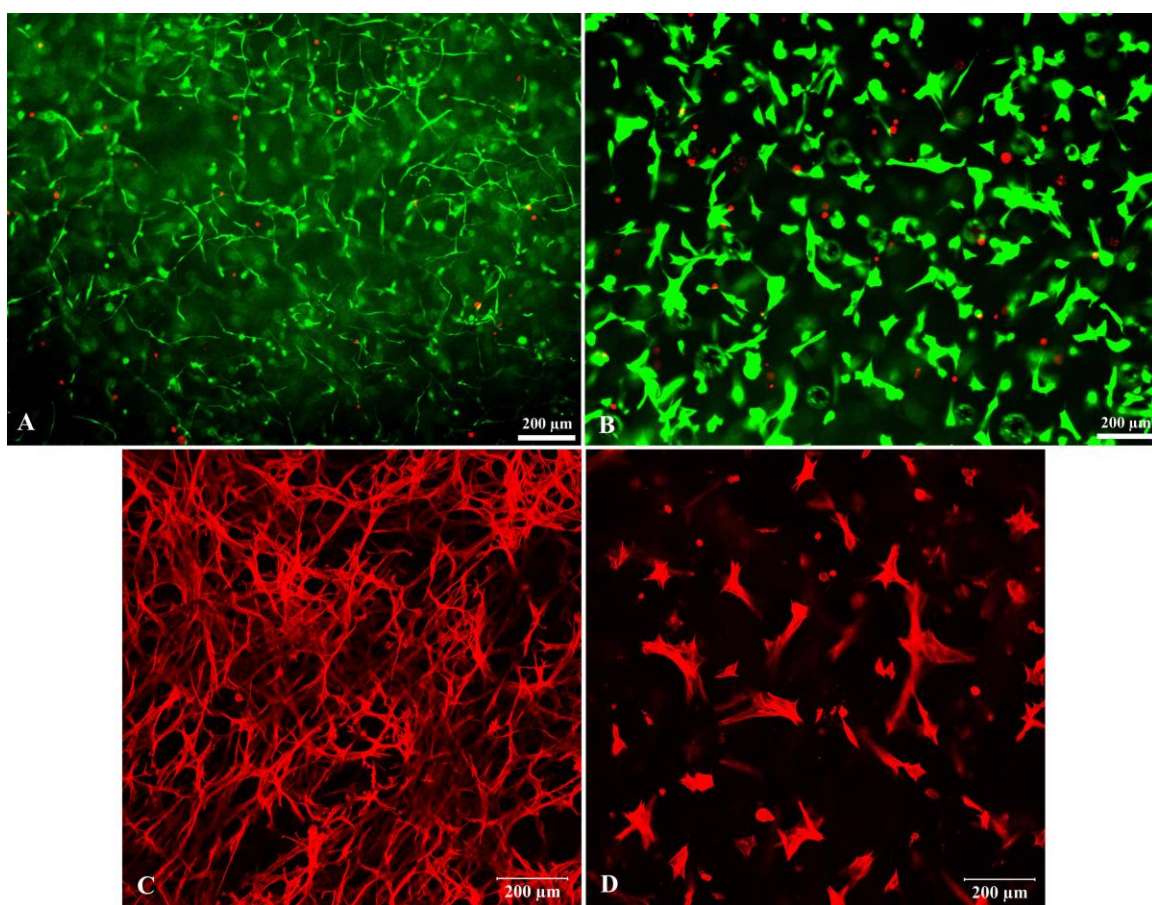


Figure 5.11 Viability and morphology of fibroblasts encapsulated in 6% w/v PEG-bis-AB/AP 50:50 blend semi-IPNs containing 0.12 % w/v HA (A) and 2% w/v GMHA hydrogels (B) visualized by staining with fluorescein diacetate/propidium iodide after 7 days in culture. Confocal analysis (200μm depth) of fibroblast morphology and spreading within 6 % w/v PEG-bis-AB/AP 50:50 blend semi-IPNs containing 0.12% w/v HA (C) and 2% w/v GMHA hydrogels after 14 days in culture (D). All scale bars represent 200μm.

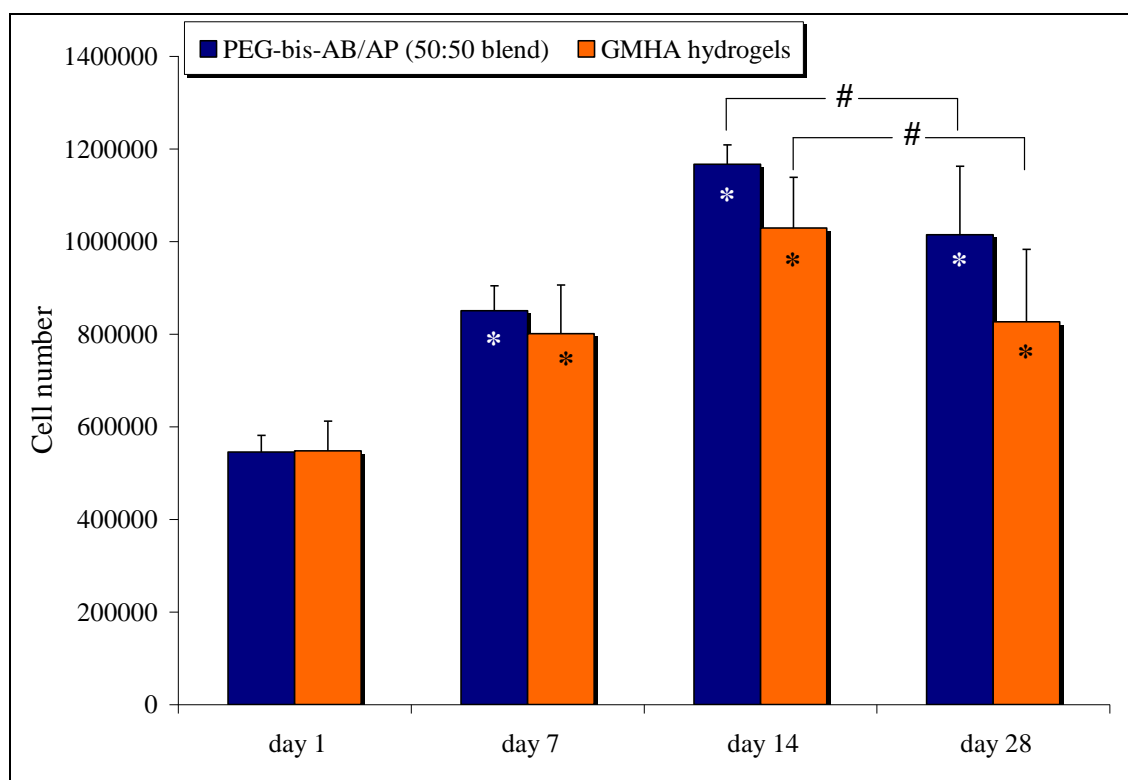


Figure 5.12 Fibroblast proliferation within 6% w/v PEG-bis-AB/AP 50:50 blend semi-IPNs containing 0.12% w/v HA (A) and 2% w/v GMHA hydrogels (B). * indicates statistically significant differences relative to the corresponding cell number at day1. # indicates statistically significant differences in cell numbers between days 14 and 28.

5.3.4 ECM synthesis

Encapsulated fibroblast synthesis of collagen (Figure 5.13) and s-GAGs (Figure 5.14) in PEG-based semi-IPNs (A,B) and GMHA hydrogels (C,D) was histologically examined at 14 (A,C) and 28 days (B,D) in culture. The ECM deposition in the PEG based semi-IPNs and the GMHA hydrogels was observed to be pericellular in nature. However, there was a noticeable difference in the spatial organization of both

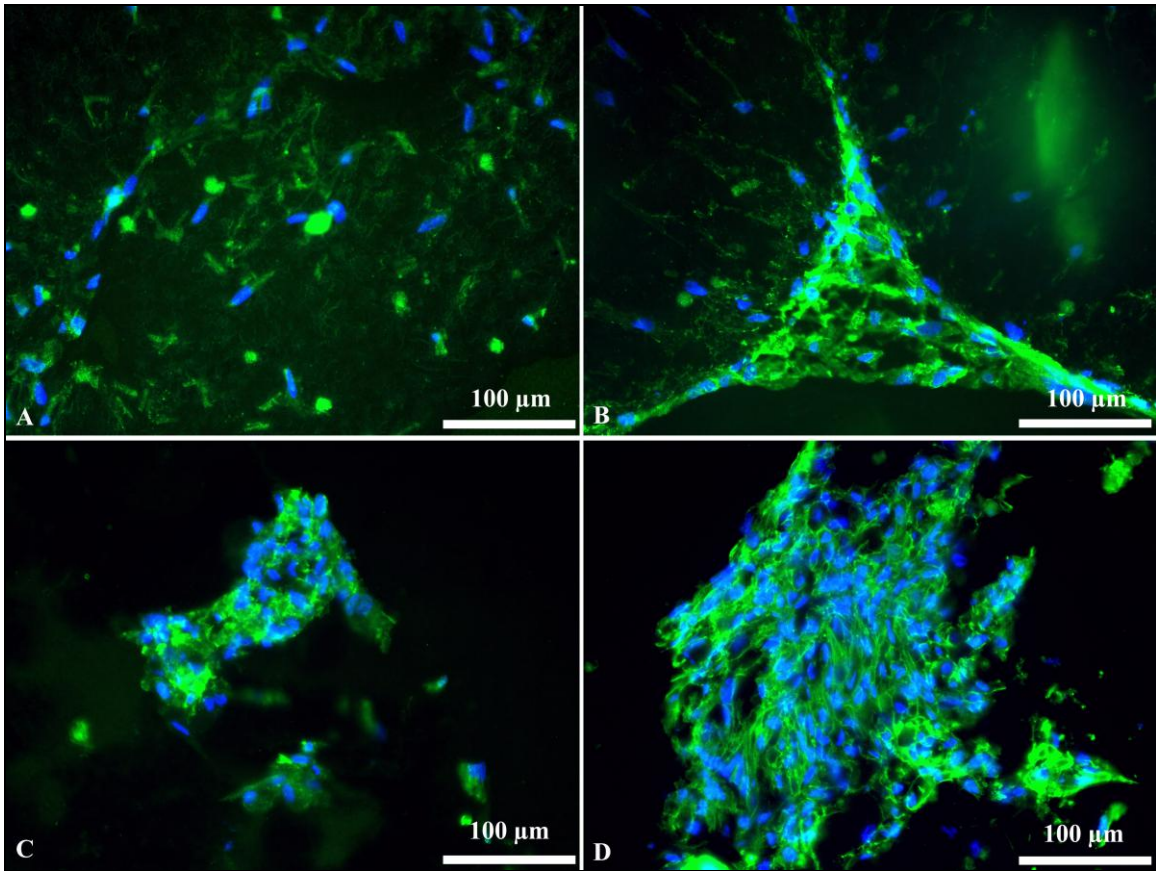


Figure 5.13 Immunohistochemical staining of collagen (green) and cellular nuclei (blue) in 6% w/v PEG-bis-AB/AP 50:50 blend semi-IPNs (A,B) and 2% w/v GMHA hydrogels (C,D) after 14 (A,C) and 28 (B,D) days in culture. All scale bars represent 100µm.

collagen and s-GAG deposition by the encapsulated fibroblasts with respect to the different materials. In the PEG based semi-IPNs, the newly synthesized matrix was relatively homogeneously dispersed, whereas in the GMHA hydrogel matrix its spatial distribution was highly variable with regions of low and high density. The fibroblasts encapsulated in the GMHA hydrogels exhibited increasing aggregation at later time points (day 28). As a result the matrix produced by these cells appears to be concentrated

at various locations in the hydrogel bulk associated with dense clusters of aggregated cells as shown by the nuclear counterstaining in Figure 5.13D.

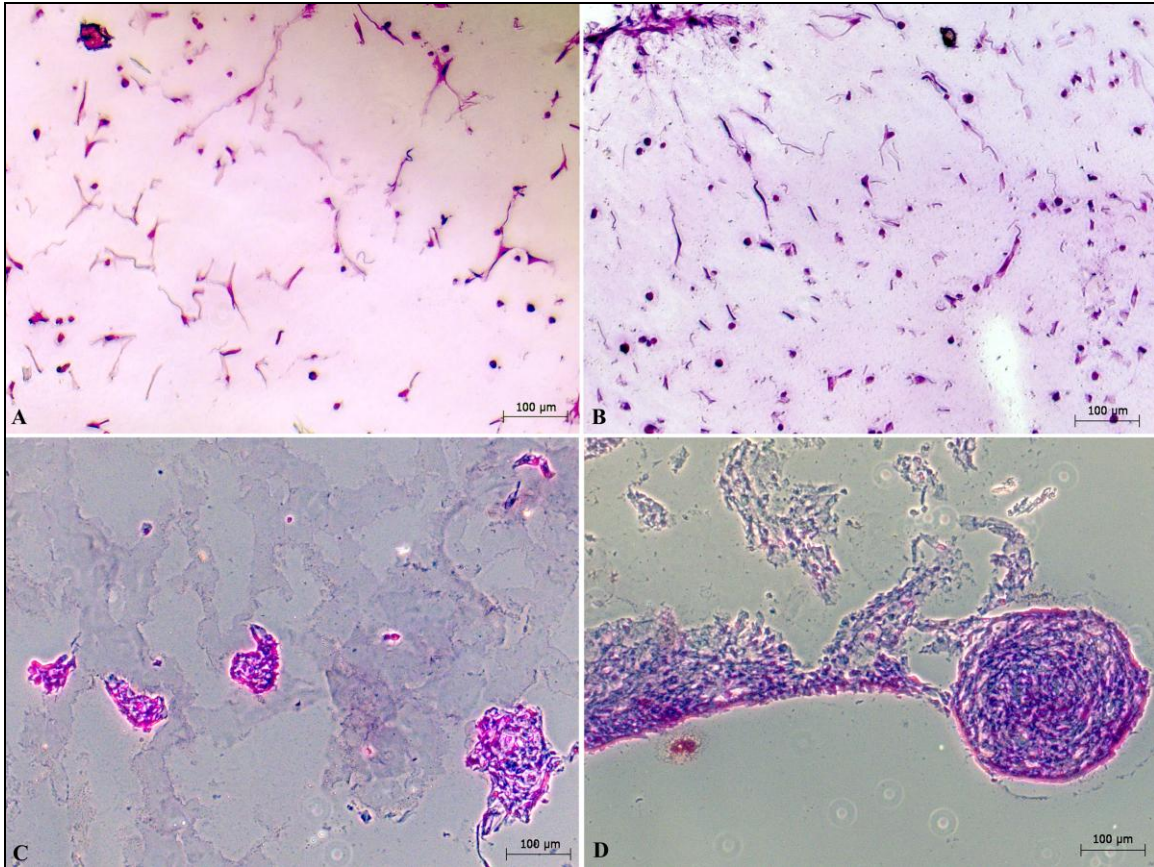


Figure 5.14 Histological staining of s-GAGs (dark purple) in 6% w/v PEG-bis-AB/AP 50:50 blend semi-IPNs (A,B) and 2% w/v GMHA hydrogels (C,D) after 14 (A,C) and 28 (B,D) days in culture. All scale bars represent 100μm.

5.4 Discussion

Vocal fold injury initiates a wound healing response resulting in alterations in ECM composition and tissue biomechanics. The functional outcome of vocal fold

scarring is dysphonia due to increased phonation threshold pressure (subglottic pressure required to initiate vibration) and reductions in vibrational amplitude of the mucosal wave. Clinical signs include decreased voice intensity, vocal fatigue, breathlessness, and increased phonatory effort [18]. Treatment of the scarred vocal folds is one of the most challenging voice problems faced by otolaryngologists today [18] since none of the current injectables and surgical techniques directly address restoration of the complex ECM composition and biomechanical properties.

At birth, the human vocal fold is a homogeneous structure lacking the complex layered organization and variable ECM composition observed in the adult [286, 287]. Recent analyses of pediatric human vocal folds from subjects ranging from near birth to adolescence indicate that the vocal folds undergo extensive postnatal development and maturation into the early teens [58, 82]. Consistent with the recognized contributions of externally applied mechanical forces in the development and homeostatic maintenance of many soft connective tissues, these results suggest that the complex mechanical forces imposed during voice development may be an important epigenetic mechanism regulating tissue ECM composition and biomechanics. As a basis for restoring structural integrity and phonation-associated mechanical stimulation to the injured vocal folds, we investigated the formulation of mechano-mimetic hydrogels approximating the mechanical properties of the two major vocal fold components, the vocal mucosa (viscous – GMHA) and the vocal ligament (elastic – PEG-diacrylate). These materials were evaluated by rheological and tensile testing methods applied to characterization of

the native tissue layers [63, 288], and tested for their ability to support encapsulated cell viability, proliferation, and ECM synthesis.

Due to favorable properties such as non toxicity, non immunogenicity, biocompatibility, and elastic mechanical properties following covalent crosslinking, PEG was chosen to restore/regenerate the elastic properties of the vocal ligament. Photopolymerized PEG-based hydrogels have also been shown to exhibit strong adherence to tissue surfaces through mechanical interdigitation and hydrogen bonding [216, 289]. Literature review suggests that the greatest stress applied to the vocal fold tissues is the longitudinal tensile stress in the vocal ligament. The mean elastic modulus of the human vocal ligament reported for the low-strain regions of the biphasic stress-strain curve was $33.1 \pm 10.4\text{kPa}$ [63]. The 6% w/v PEG-bis-AB hydrogels provided an elastic modulus of $26.4 \pm 3.65\text{kPa}$. Though the 6% w/v PEG-bis-AB hydrogels provided an elastic modulus which approximated that of the human vocal ligament at low strain levels (0 – 15%) it did not show a biphasic stress-strain curve which is characteristic of the human vocal ligament [63, 277] and other soft tissues [285]. The 6% w/v PEG-bis-AB hydrogels exhibited a linear stress-strain curve till failure at $46 \pm 2\%$ strain. The linear and non linear patterns exhibited at low and high strains correlate to the unique collagen ultrastructure of the human vocal ligament [113]. The helical collagen fibers function as a buffer at low strains so that elongations of the vocal ligament occur without much resistance [113]. At high strains, the unique collagen structure absorbs the stress and serves as a shock absorber along the length of the fibers. The lack of a biphasic stress-strain curve may be perceived as a limitation of these PEG-based hydrogels.

However, since the vocal fold operates at low strain levels (0 – 15% strain) [8] during normal phonation we propose the use of the 6% w/v PEG-based hydrogels during the acute phase of wound healing during which the patient may be advised against strenuous vocal activity. It must be noted that only the PEG-bis-AB hydrogels were subjected to uniaxial tensile testing whereas the PEG-bis-AP/AB blends were used for cell culture studies. The only difference between the 6% w/v PEG-bis-AP, PEG-bis-AB, and PEG-bis-AP/AB hydrogels is the rates of degradation. We have previously reported that a slight variation in chemistry that causes significant differences in degradation rates does not affect mechanical properties of these PEG-based hydrogels [245]. The relatively small mesh size of hydrolytically degradable PEG-based hydrogels initially restricts cell spreading which may be detrimental to cell growth and tissue formation. We have previously shown that incorporation of 0.12% w/v HA substantially increases cell spreading and proliferation without significantly altering the elastic properties [282].

The abundance of HA in the vocal mucosa imparts viscous shear properties to the vocal fold tissue, and helps produce and sustain vibration over a vast range of frequencies (100 – 1000Hz) [7]. HA also plays a vital role in wound healing [269]. In the acute/proliferative phase of adult wound healing, fibroblasts migrate into the site of injury, and actively synthesize ECM consisting mainly of type I collagen [126]. The balance between matrix macromolecules (collagen vs HA) produced during the proliferative phase is one critical difference between scar formation (adult wounds) and regenerative-type wound healing (early fetal wounds) [128]. HA being a space occupying molecule in the ECM, influences collagen fibrillogenesis [127]. Adult dermal

wound healing is characterized by scar formation attributed to decreased/degraded HA levels by day 10 [129]. Scarless fetal wound healing is attributed to consistent elevated levels of HA leading to regenerative repair [128, 130, 269]. It has been reported that HA levels in the vocal fold wounds decrease to subnormal levels after day 5 of injury resulting in vocal folds scarring [131]. Therefore, our GMHA hydrogel scaffolds may help to sustain HA levels throughout the wound healing process, and potentially reduce scar formation through regulating collagen fibrillogenesis. On comparing our data with literature studies of the viscous shear properties of the human vocal mucosa, we observed that the magnitude of dynamic viscosity of the 1.5 – 2.5% GMHA hydrogels at all the tested frequencies lies in the same range as that of the human vocal mucosa [103, 195, 276, 288]. The broad range of the magnitude of dynamic viscosity of the vocal mucosa (100 – 1000Pa.s) has been attributed to intersubject differences in sex and age [288]. However, the elastic shear modulus of the 1.5 – 2.5% GMHA hydrogels at all the tested frequencies is slightly higher than the average values reported for the human vocal mucosa [276]. This may not be perceived as a limitation of the GMHA hydrogels since the elastic shear modulus data reported for the human vocal ligament has a high degree of variability.

Rheological studies indicated an order of magnitude difference among the magnitude of dynamic viscosities of the 2% w/v unmodified HA solution, 2% w/v GMHA solution, and 2% w/v GMHA hydrogels at all the frequencies tested. This suggests a decrease in the molecular weight of the GMHA macromer (in comparison to native unmodified HA), likely attributable to degradation during the methacrylation

process. The degree of reduction in the molecular weight of the GMHA conjugates was not quantified using other methods since it is beyond the scope of the present study, and subsequent covalent crosslinking achieved mechanical properties approximating the native vocal mucosa. Rheological tests were not performed at physiologically relevant frequencies (>100 Hz) since at these frequencies the inertial effects of the sample and the rheometer's plate become significant due to which the stress-strain linearity cannot be maintained. However, it has been previously reported that low frequency data can be extrapolated to physiologically relevant frequencies based on constitutive modeling or time-temperature superposition [276].

Data from our degradation tests as well as previous studies [245] demonstrated the ability of PEG-based macromers synthesized with chemical intermediaries containing variable length alkyl spacers to form hydrogels with controlled variation in hydrolytic degradation rate. At 6% w/v, the PEG-bis-AP and PEG-bis-AB compositions and their 50:50 blend provided degradation times ranging from 7 – 18 weeks. Previous studies using naturally-derived matrices have observed 2 – 3 week time-frames for *in vivo* degradation following implantation into animal models of vocal fold injury [42, 43]. As the optimal kinetics of scaffold remodeling and degradation has not been fully determined, the synthetic component described here for the vocal ligament offers a range of possibilities. The mass loss of GMHA hydrogels was not characterized unlike the PEG-based hydrogels since these HA based hydrogels are subject to enzymatic (HAse) degradation which depends on the number of cells encapsulated within the hydrogels. Any mass loss studies conducted with encapsulated cells would confound results due to

the presence of cells and neo-matrix produced by the encapsulated/proliferating cells. However, the enzymatic degradation of GMHA hydrogels in the presence of HAs has been previously reported by Leach et al [289, 290]. In case of our proposed in vivo application the degradation of the GMHA hydrogels solely depends on the amount of HAs produced by the transplanted/endogenous cells.

A significant increase in encapsulated fibroblast number at days 7 and 14 in both 6% PEG-based semi-IPNs and 2% w/v GMHA hydrogels indicated the cytocompatibility of these materials. The reduction in cell number at day 28 can be attributed to semi-IPN/hydrogel degradation, which resulted in the release of cells and their adhesion to the underlying culture substrate (data not shown). Histological and immunohistochemical analysis demonstrated that both 6% w/v PEG-based semi-IPNs and 2% w/v GMHA hydrogels supported the synthesis and accumulation of type I collagen and s-GAGs. Since the final application involves the use of the 2% w/v GMHA hydrogel as a scaffold for the vocal mucosa that is comprised mainly of proteoglycans/GAGs, the production of collagen in these materials may be viewed as a negative observation. This result suggests that tissue-specific mechanical properties may not be sufficient to completely direct tissue-specific ECM production by the encapsulated cells. However, previous work showing that externally applied vibration significantly upregulated MMP-1 and HA synthase 2, but not type I collagen suggests that vibratory stimulation may be an effective tool in combination with these materials to more effectively regulate ECM composition [11].

5.5 Conclusion

These studies demonstrate that appropriate formulations of chemically modified PEG and HA can produce hydrogel matrices with mechanical properties consistent with the native human vocal mucosa and ligament. These degradable matrices may be crosslinked in the presence of cells and support cell viability, proliferation, and ECM synthesis. Bearing in mind the slight limitations of these materials and the significance of mechanical forces associated with phonation, it is expected that physiologically relevant mechanical stimulation will further improve scaffold remodeling and tissue-specific ECM expression. Future studies will investigate the gene and protein expression of fibroblasts encapsulated in 2% w/v GMHA hydrogels, cultured in a vibrational bioreactor at physiologically relevant frequency and amplitude.

CHAPTER 6

THE EFFECT OF VIBRATION ON GENE AND MATRIX EXPRESSION OF FIBROBLASTS ENCAPSULATED IN HYALURONIC ACID HYDROGELS

6.1 Introduction

Voice is critical to the quality of life and is the primary “tool of trade” for 25% of the US work force (teachers, singers, sales personnel etc) [1]. Phonation (voice production) is a co-ordinated biological process involving the vocal folds, lungs, brain, peripheral nervous system, and muscles of the respiratory tract synergistically transducing aerodynamic energy from exhalation to acoustic energy. The vocal folds are the dynamic mechanical element of the phonatory system and possess a spatially organized ECM composition that is fundamental to the tissue biomechanics and in turn the quality of voice [2]. The human vocal folds are multi-layered, consisting of a squamous epithelium, lamina propria composed of fibroblasts and ECM, and the thyroaretenoid muscle. The squamous epithelium and the superficial lamina propria together form the *vocal mucosa*. The vocal mucosa is abundant in HA, small proteoglycans like fibromodulin and decorin, and large proteoglycans like versican that regulate the water content of the tissue and impart viscous shear properties to it [2-4, 92]. The intermediate and deep layers of the lamina propria together form the *vocal ligament*. The intermediate layer is abundant in elastin fibers which impart flexibility and distensibility to the vocal fold tissues during repeated deformation from phonation [2, 3, 92, 110]. The deep layer is abundant in collagen fibers which are relatively inextensible,

resist and transmit tensile stresses and are responsible for the maximum dimensions of the vocal fold tissues [2, 92, 119]. The vocal mucosa is the vibratory element of the structurally/functionally differentiated vocal fold tissue that sustains high frequency vibration during phonation. The human vocal fold mucosa vibrates at frequencies of 100 – 1000Hz, at amplitudes of ~1mm during phonation [5]. Vocalization times can vary between 1 to 2hrs per day for heavy voice users like classroom teachers and opera singers [9-12].

The most commonly/severely afflicted site in terms of vocal pathologies is the vocal mucosa. Injuries to the vocal mucosa brought about by chronic vocal tissue overuse, laryngeal cancer, chemical or thermal injury, non phonatory mechanical trauma, and attempted repair lead to a wound healing response which is characterized by conversion of the GAG-based tissue to fibrous scar tissue [13-15, 123]. Structural changes due to changes in ECM composition of the vocal mucosa lead to altered vibratory biomechanics which ultimately causes dysphonia. Dysphonia characterized by increased effort to sustain phonation, decreased vocal quality, and total voice loss affects approximately 23% of the voice-dependent US workforce [16]; and may jeopardize job security, employment opportunities, and social interaction [225, 226]. It is estimated that approximately 9% of the population suffers from voice related problems at any given point in time [15]. Scarring induced dysphonias are currently treated by voice therapy and surgical augmentation procedures. Current treatment techniques, although effective in improving short term voice quality, have been unable to achieve complete restoration

of normal voice due to their inability to bring about regeneration of the native vocal fold tissue architecture/ECM composition.

The ECM composition and biomechanical properties of many human tissues are regulated by their mechanical environment during both developmental morphogenesis and adult homeostasis [50, 51]. Using 3D culture systems and bioreactors, application of physiologically relevant mechanical stimulation has been shown to elicit tissue-specific ECM expression and increases in corresponding mechanical properties relative to static controls. Examples include application of cyclic strain to smooth muscle cells [291] and fibroblasts [292], and dynamic compression/hydrostatic pressure to chondrocytes [293, 294]] and annulus fibrosus cells [295, 296]. While the effects of cyclic strain/compression have been widely investigated, the response of cells to vibratory stimulation has been relatively less explored. Tanaka and co-workers reported increased expression of osteocalcin and MMP-9 by osteoblasts cultured in collagen gels subjected to various vibratory regimes relative to static controls [55]. Titze and co-workers [11] observed significant increases in mRNA expression of proteoglycan/GAG genes in tracheal fibroblasts cultured under vibratory stimulation within 3D, porous, elastomeric tecoflex substrates.

Our long-term hypothesis is that rapid restoration of the vibratory microenvironment using mechano-mimetic scaffolds will facilitate vocal mucosa-specific ECM deposition by the resident/infiltrating cells, ultimately leading to improved functional outcomes by reducing fibrotic scarring during the acute stage of wound healing. Towards this end, we have previously reported the preparation of injectable

GMHA hydrogels with rheological properties approximating those of the native human vocal mucosa [297]. However, fibroblasts encapsulated within GMHA hydrogels synthesized an ECM rich in both collagen and s-GAGs under static conditions. The objective of this study was to investigate the ability of physiologically relevant high frequency vibration to stimulate vocal mucosa-like gene expression and ECM accumulation by human fibroblasts encapsulated in mechano-mimetic GMHA hydrogels.

6.2 Materials & Methods

6.2.1 Materials

Sodium hyaluronate ($MW = 1.4 \times 10^6$) was purchased from Genzyme (Cambridge, MA). Polyethylene glycol diacrylate (PEGDA) ($MW=258$) and tetrabutylammonium bromide, triethylamine, and glycidyl methacrylate were obtained from Aldrich (St. Louis, MO, USA). Dichloromethane (HPLC grade) was obtained from Acros organics (NJ, USA). Acetone was purchased from Fisher Chemical (Fair Lawn, NJ). 2-hydroxy-1-(4-(hydroxyethoxy) phenyl)-2-methyl-1-propanone (I-2959) was obtained from Ciba Specialty Chemicals (Basel, Switzerland). A 10% w/v stock solution of I-2959 was prepared in 70% ethanol. Adult normal human dermal fibroblasts (NHDF, CC-2511) obtained from a 39 year old Black woman were purchased from Biowhitaker (Rockland, ME, USA). DMEM F-12 50/50, 1X with L-glutamine, 15mM HEPES/trypsin EDTA (0.05% trypsin/0.53mM EDTA in HBSS), and penicillin/streptomycin were obtained from Mediatech (Herdon, VA, USA). Bovine growth serum was purchased from Hyclone (Logan, UT, USA).

6.2.2 Synthesis of GMHA

GMHA was synthesized by reacting sodium hyaluronate solubilized in double distilled water with a 20-fold molar excess of glycidyl methacrylate in the presence of excess triethylamine and tetrabutylammonium bromide as previously described [280]. The GMHA was precipitated in acetone, dialyzed against double distilled water for 48 hours, and recovered by lyophilization. An ~7% degree of methacrylation was calculated from the $^1\text{H-NMR}$ (D_2O) spectra based on the ratio of peak integrals derived from the methacrylate and HA carbohydrate protons, as previously described [281].

6.2.3 Cell culture

NHDF were routinely cultured in 175 cm^2 T-flasks using DMEM F-12 50/50, 1X with L-glutamine and 15mM HEPES enriched with 10% v/v bovine growth serum and 50U/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin. Culture medium was changed once every two days and cells were passaged weekly. All experiments were performed with cells from the 4th – 6th passages. For hydrogel encapsulation, monolayers of fibroblasts (ca. 95% confluence) were trypsinized, centrifuged, resuspended, counted in a hemacytometer, and adjusted to a final concentration of 1.6×10^7 cells/ml.

6.2.4 Bioreactor construction

A vibrational bioreactor module comprising a modified 75 cm^2 T-flask and a voice coil actuator (BEI Kimco) was constructed partly as explained previously [11]. The vibrational bioreactor assembly is shown in figure 6.1. A sinusoidal waveform from a function generator (BK Precision, CA) was fed to the input terminal of a reed switch.

The reed switch was connected at the collector terminal (+5V from a PC) of a NPN transistor, the base terminal of which was connected to the output from a custom Labview program (National Instruments) and an analog output board running on a PC. The variables controlled by the Labview software were the total number of work days, active vibration intervals (*on* and *off* times) and inactive (rest) time per day. The output from the reed switch stimulated the voice coil which ultimately controlled the vibration actuator bar. The voltage level of the sinusoidal wave was set so as to maintain 1mm vibrational amplitude at the actuator bar which was verified using a digital stroboscope before every experiment.

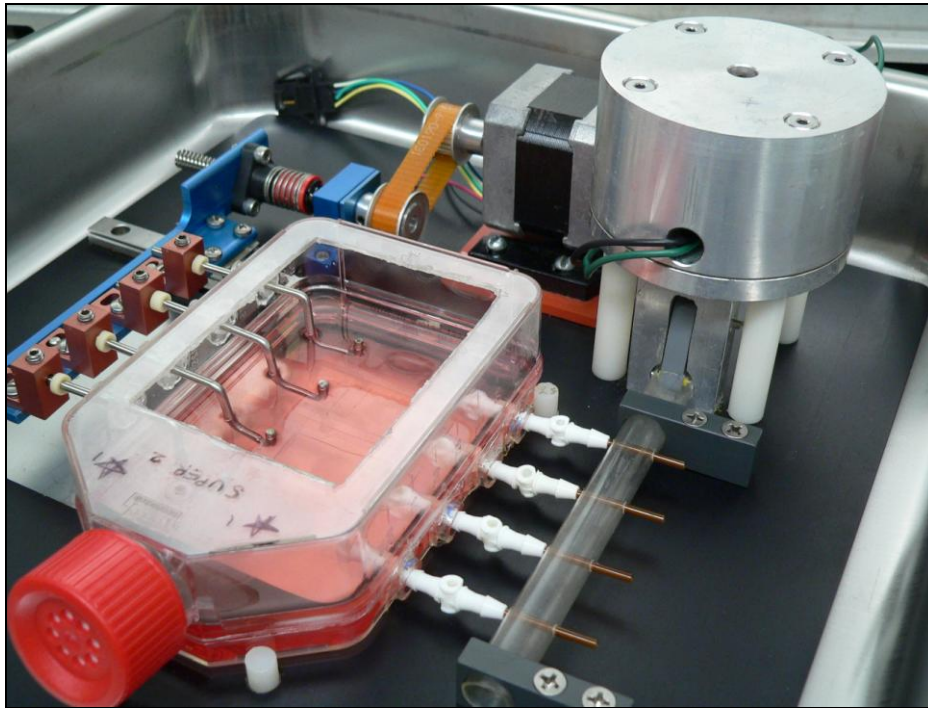


Figure 6.1 A vibrational bioreactor module with a modified 75cm² T-flask.

6.2.5 Bioreactor cell culture

6.2.5.1 Tecoflex film fabrication

The cell encapsulated hydrogel samples were crosslinked to elastomeric Tecoflex films mounted on “U” shaped plastic frames (40mm x 15mm). Briefly, Tecoflex SG-80A (Thermedics) was dissolved in methylene chloride at a 5% concentration and films were made by solvent casting. The Tecoflex films were subjected to oxygen plasma treatment for 8 mins at 300mTorr after which they were immersed in PEGDA (MW=258) for 30 mins to introduce free vinyl groups on the film surface.

6.2.5.2 Hydrogel photopolymerization and vibrational culture

4% w/v GMHA macromer solution was prepared in sterile 1X-PBS (0.1M, pH 7.4). A fibronectin-derived cell adhesion peptide, (GRGDS, Bachem, PA, USA) was conjugated to acrylate-PEG-NHS (MW=3400, Nektar) as previously described [216]. GRGDS-PEG-acrylate was prepared as a stock solution in 1X PBS (10 μ mol/ml) and sterile filtered. For cell encapsulation studies, the final concentrations of GMHA, acrylate-PEG-GRGDS, and NHDF were 2%, 2.5 μ mol/ml, and 4x10⁶cells/ml, respectively, unless mentioned otherwise. The cell-macromer-peptide suspension (200 μ l) containing 0.1% w/v I-2959 initiator was crosslinked on the modified Tecoflex film by exposure to low intensity UV illumination (365nm, 10mW/cm², Black-Ray B100-AP, Upland, CA) for 6½ minutes (Figure 6.2A). N=4 hydrogel samples were mounted between the support pins in the bioreactor flask (figure 6.2B, C and D) and the frames cut to allow the samples to move freely in the axial and longitudinal directions.

The hydrogel samples were allowed to equilibrate to the new environment overnight before the vibratory stimulus was applied. The samples were then stimulated by a 100Hz, 5.3Vrms sinusoidal waveform from a function generator in a 2sec *on*/2sec *off* regimen for 4hours/day. N=4 fibroblast encapsulated hydrogel samples cultured in a Petri dish under static conditions served as controls.

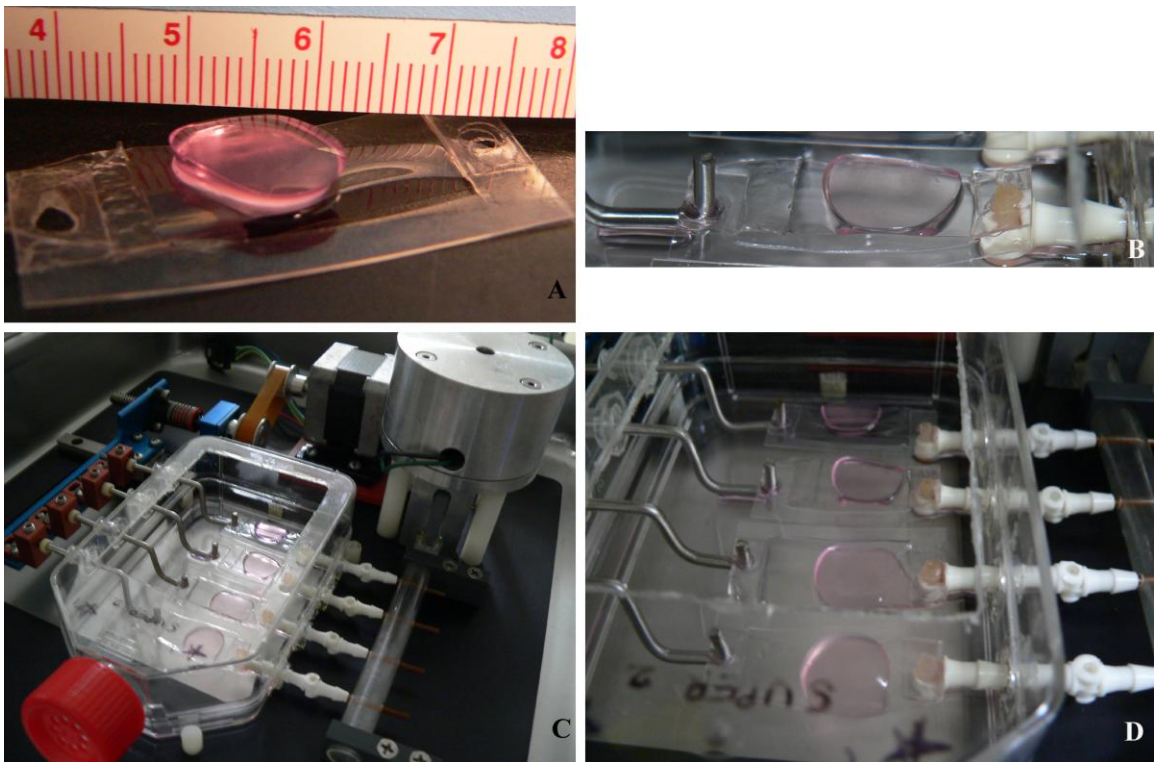


Figure 6.2 A 2% w/v GMHA hydrogel encapsulated with cells and immobilized on an elastomeric Tecoflex film by photopolymerization (A), different views of GMHA hydrogels mounted in a modified 75cm² T-flask of the vibrational bioreactor module (B), (C), and (D).

6.2.6 Encapsulated fibroblast viability, morphology, and proliferation within the 2% w/v GMHA hydrogels

6.2.6.1 Viability assessment

After 5 and 10 days in culture, hydrogel samples (N=4/time point) from both the vibrational and static groups were collected from the study, rinsed three times with sterile 1X-PBS, and stained with 1 μ M fluorescein diacetate (FDA, Molecular Probes, Eugene, OR) and 2.5 μ M propidium iodide (PI, Molecular Probes). The samples were once again rinsed with sterile 1X-PBS, visualized, and imaged by fluorescence microscopy (Zeiss Axiovert 200).

6.2.6.2 Confocal analysis of morphology

After 1, 3, 5, and 10 days in culture, samples (N=4/time point) from both the vibrational and static groups were fixed in 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100, and stained with Alexa 594-phalloidin (Molecular Probes). Samples were visualized and imaged using a Zeiss Confocal LSM510 microscope.

6.2.6.3 Fibroblast proliferation

N = 4 hydrogel samples each from the vibratory and static groups were collected at days 1, 5, and 10, rinsed twice with sterile PBS, snap-frozen in an ethanol-dry ice bath, and stored at -80°C. DNA solubilization and quantitation were based on modifications of previously described methods [247]. Briefly, the samples were subjected to 3 freeze-thaw cycles in 1.4ml of 10mM EDTA, pH 12.3. Once the hydrogels were completely

dissolved, the pH was neutralized by the addition of 100µl 1M potassium phosphate (monobasic) solution. Total DNA content was measured using the Picogreen DNA-binding dye (Molecular Probes) and a fluorescence microplate reader (Tecan GENios, excitation: 485nm, emission: 535nm). DNA content was converted to cell number based on a DNA standard curve prepared from serial dilutions of NHDF and compared to the cell number at the end of day 1.

6.2.7 Gene expression

The hydrogel-encapsulated fibroblast samples (N=4 samples/time point) from both the vibrational and static groups were harvested from culture after 1, 3, 5, and 10 days; and stored in 1ml Trizol reagent (Aldrich) at -80 °C. The frozen hydrogel samples were thawed, homogenized, centrifuged at 13,000g for 15mins at 4°C, and the supernatant removed and collected in clean PCR tubes. Total RNA was isolated according to the manufacturer's protocol and finally redissolved in 20µl RNase-free water. The samples were treated with RNase-free DNase I (Turbo, Ambion) to eliminate genomic DNA contamination. Using a UV spectrophotometer, concentration and purity of the total RNA was determined based on absorbance at 260nm and 280nm. Samples containing 1µg of total RNA was reverse transcribed to cDNA in a 20µl reaction using the Retroscript cDNA Synthesis Kit (Ambion), and the Rotorgene 3000 light thermal cycler (Corbett Research, Mortlake, NSW, Australia). Realtime PCR was performed using custom-designed sense and anti-sense primers (Table I) and the QuantiTect SyBr Green PCR kit (Qiagen). Briefly, after an initial heat activation at 95°C

for 15 minutes, cDNA products were amplified through 35 cycles, each consisting of a denaturation step at 94°C for 15 seconds, an annealing step at 54°C for 20 seconds, and an extension step at 72°C for 20 secs. Melt curve analysis was performed at the end of all reactions to verify formation of single product. Amplification efficiencies were tested using serial dilutions of cDNA covering four orders of magnitude and found to range from 0.94-1 for all products. Relative expression levels of target genes were quantified by the $\Delta\Delta C_T$ method using $\beta 2$ microglobulin ($\beta 2M$) as an internal control and the day 1 static samples as reference condition (calibrator) [298]. Data for days 3 and 5 represent the combined results of two independent experimental replicates.

Target gene	Accession ID	Sense primer	Antisense primer
Collagen 1	NM_000088.2	Tgctggtgctcctgttactc	tccagagggaccttgtttgc
Elastin	NM_000501.1	Gcctacaccacagggaaac	accgaacttgctgctgctt
HA Synthase 2	NM_005328.1	cataaagaaagctcgcaacacg	cacacttcgtcccagtgctc
Decorin	NM_001920.3	Ggtcagccggattgtgttc	gcaggtgtggaaaggaggag
Fibromodulin	NM_002023.3	Tgataaggtgggcaggaagg	tggttggtgctgagatggag
MMP-1	NM_002421.2	Tgctgaaaccctgaaggtga	cttggaatctggcgtgta
Beta-2-microglobulin	NM_004048.2	Tgtgctcgcgtactcttc	cggatggatgaaaccagac

Table 6.1 Target Genes and Primers for Real Time RT-PCR Analysis.

6.2.8 Encapsulated fibroblast ECM production

In order to quantify ECM production by the encapsulated fibroblasts, N=4 samples/time point from both the vibrational and static groups were collected from the study after 5 and 10 days of culture. The samples were digested using 1ml Hase (40U/ml) per hydrogel under constant agitation for 4hours. Following digestion, the samples were transferred to 2ml tubes, and stored at -80°C.

6.2.8.1 s-GAG synthesis (DMMB Assay)

s-GAG accumulation was measured using the 1,9-dimethylmethylene blue (DMMB) assay as previously described [299]. Briefly, the assay was performed by adding 20µl of the sample to 200µl of the DMMB reagent stock solution (16mg DMMB + 5ml 95% ethanol + 3ml formic acid + 25.6ml NaOH + 966.4ml distilled water, pH 3.5) in a 96-well plate. DMMB dye binding to s-GAGs produced a pink color which was measured by absorbance at 525 nm using a microplate reader (Bio-Tek Instruments, Winooski, VT) and was quantified relative to a linear standard curve prepared from chondroitin-4-sulfate standards (0 – 100µg/ml). The absorbance of cell-free 2% w/v GMHA hydrogels was subtracted from the absorbance values of vibrational/static groups in order to account for absorbance of the GMHA material alone. For data analysis, s-GAG values were normalized to total DNA values.

6.2.8.2 Collagen synthesis (Hydroxyproline analysis)

Collagen accumulation by the encapsulated fibroblasts in both the vibratory and static groups was quantified by the hydroxyproline assay as previously described [300]. The total collagen content was calculated based on the estimation that hydroxyproline makes up about 13.2% of the total collagen [301]. The total collagen values were normalized to total DNA values.

6.2.9 Rheological characterization of cell-free and cell-encapsulated 2% w/v GMHA hydrogels following 10 day vibrational bioreactor culture

In order to investigate the effect of the vibrational regimen and neo matrix synthesis on the mechanical properties of the 2% w/v GMHA hydrogels, two cell-encapsulated hydrogel groups (4×10^6 cells/ml and 2×10^6 cells/ml), and cell-free hydrogels were photopolymerized and exposed to vibratory stimulation for 10 days. The hydrogel samples (vibrational samples and static controls) from both the cell-encapsulated and cell-free experiments were collected after 10 days in culture, rinsed two times with 1X-PBS sterile PBS, and immediately subjected to rheological testing under conditions similar to those used to investigate the rheological properties of the human vocal mucosa [12]. Rheological characteristics as a function of mechanical stimulus and presence/absence of cells were investigated in a stress controlled “ARES” Rheometer using parallel plate geometry. The hydrogel samples were placed in the gap (0.8mm) between the stationary upper plate and the rotating lower plate, and the temperature was maintained at $37 \pm 0.1^\circ\text{C}$ by a “Peltier” water cooling system. In order to determine the

region of linearity, strain sweep tests were performed at fixed oscillation frequency (1Hz). Rheological tests were performed in the linear strain region (strain amplitude – 0.01rad), over a frequency range of 0.01 – 15Hz, covering 25 frequencies over 3 decades. The dynamic viscosities of the 2% w/v GMHA hydrogels tested were calculated by dividing the observed values of the loss moduli by the corresponding angular frequencies tested. The elastic shear moduli of the 2% w/v GMHA hydrogels tested were also recorded and plotted at all frequencies tested.

6.2.10 Statistical analysis

All data sets are represented in terms of means \pm standard deviation with n=4. All data sets were statistically compared by ANOVA using Tukey's method for post hoc testing with "p" values less than 0.05 considered significant.

6.3 Results

6.3.1 Encapsulated fibroblast viability, morphology, and proliferation in 2% w/v GMHA hydrogels

Fibroblasts were encapsulated within 2% w/v GMHA hydrogels containing immobilized RGD cell adhesion peptides by photopolymerization and cultured under static or vibratory conditions for up to 10 days. Encapsulated fibroblast viability was observed to be substantially greater in the static controls at day 5 (Figure 6.3A) and day 10 (Figure 6.3C) than in the corresponding vibrational samples (Figures 6.3B and D). Cell viability was largely uniform throughout the bulk of the hydrogel constructs.

Fibroblasts in the static controls exhibited clustering and aggregation (Figure 6.3C) in various areas throughout the bulk of the hydrogel constructs to a much higher degree than in the vibrational samples (figure 6.3D) at the same time point.

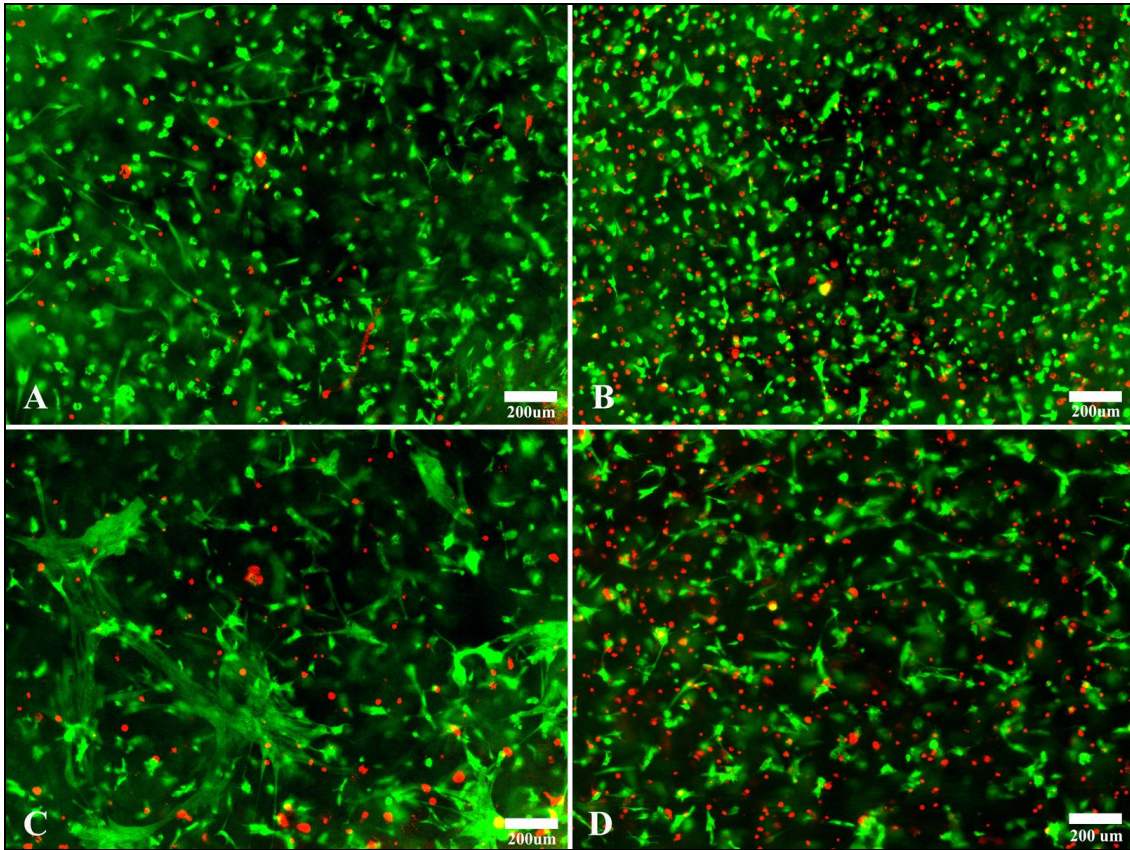


Figure 6.3 Viability of fibroblasts encapsulated in 2% w/v GMHA hydrogels and cultured under static (A,C) and vibratory (B,D) conditions for 5 (A,B) and 10 days (C,D). Cells were stained with fluorescein diacetate (green)/propidium iodide (red). All scale bars = 200µm.

Confocal microscopy was performed to visualize fibroblast morphology within the 3D hydrogel networks. Encapsulated fibroblasts exhibited spreading within 3 days (data not shown) and development of stellate morphology at day 5 in the static controls (Figure 6.4A) and the vibrational samples (Figure 6.4B). Stellate cell morphology persisted for the entire duration (day 10) of the experiments in the static (Figure 6.4C) and the vibrational samples (Figure 6.4D).

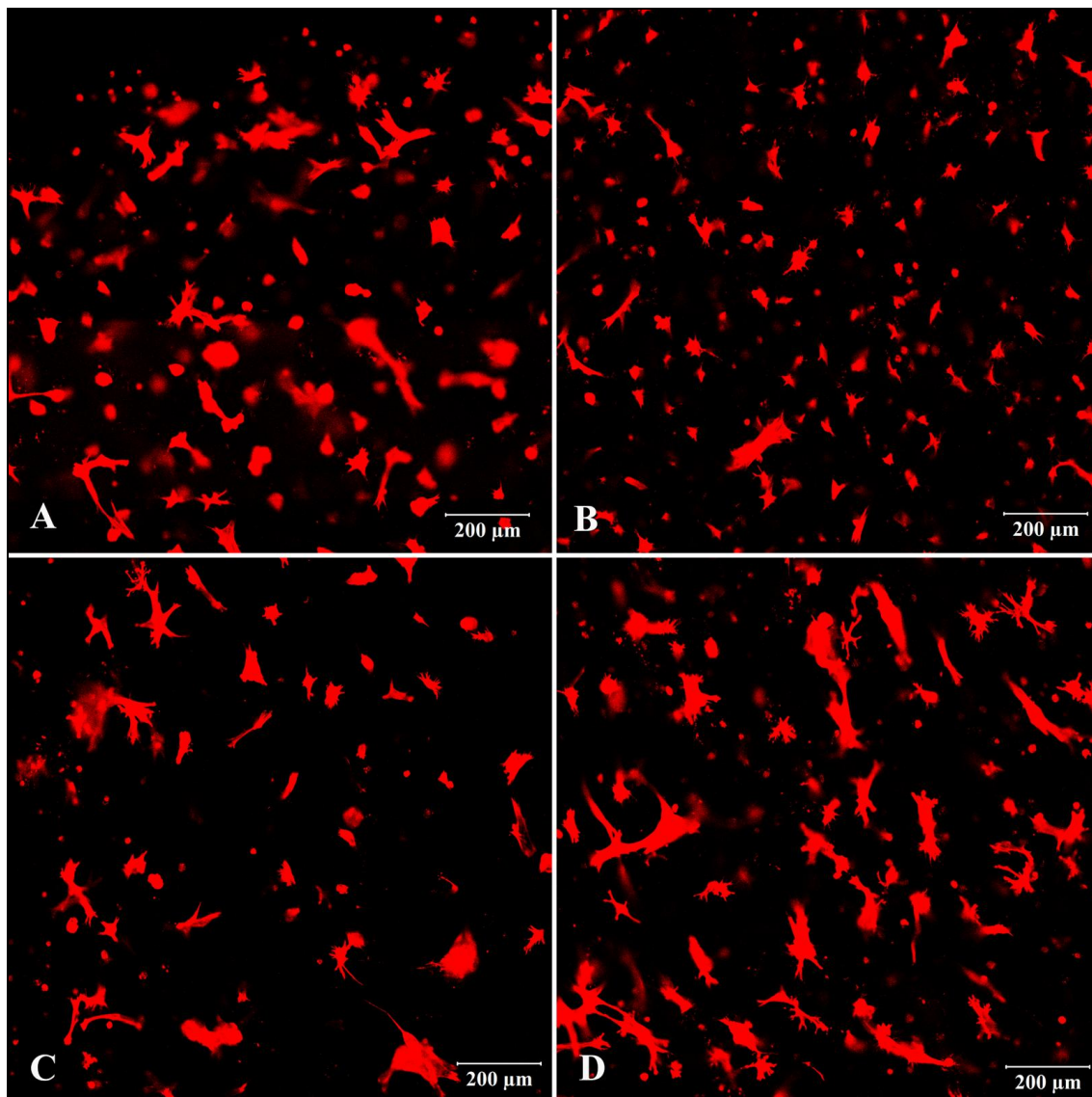


Figure 6.4 Confocal microscopy images (200 μ m depth) of fibroblast morphology and spreading within 2% w/v GMHA hydrogels. Samples were stained with Alexa 594-phalloidin and imaged after culture under static (A,C) and vibratory (B,D) conditions for 5 (A, B) and 10 days (C,D). All scale bars = 200 μ m.

The cell number (DNA content) of the hydrogel constructs at days 5 and 10 is shown in figure 6.5. Relative to the initial cell number measured at day 1, significant increases in cell number were observed in 5 day static controls and 10 day static and vibrational groups. Although cell proliferation was observed in the 10 day vibrational group (relative to day 1), cell numbers measured in vibrational samples were significantly lower than corresponding static controls at both time points (days 5 and 10) assayed.

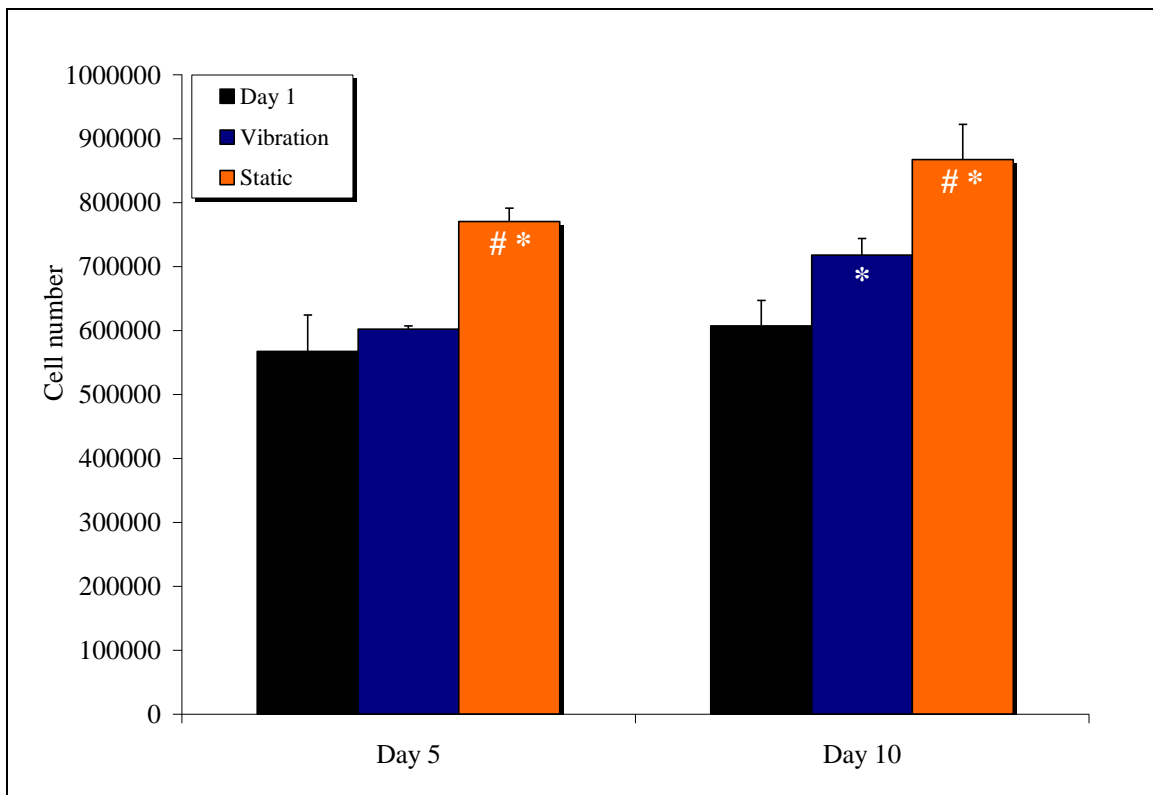


Figure 6.5 Time course fibroblast proliferation within the 2% w/v GMHA hydrogels under vibratory stimulus and static conditions. * indicates significant differences relative to the corresponding cell number at day 1. # indicates significant differences relative to the corresponding static control at the same time point.

6.3.2 Gene expression

Fibroblast mRNA expression levels of several ECM-related genes relevant to the human vocal mucosa were examined after 1, 3, 5, and 10 days of culture under vibratory and static conditions. In general, fibroblasts exposed to vibratory stimulation exhibited increased expression of HA Synthase 2 (Figure 6.6A), decorin (Figure 6.6B), fibromodulin (Figure 6.7A), and MMP-1 (Figure 6.7B) relative to static controls for corresponding time points at days 1, 3, and 5. In addition, significant temporal increases in HA synthase 2 (day 3 and 5), fibromodulin (day 3), and MMP-1 (day 5) were observed relative to day 1 among sample groups exposed to vibration. Interestingly, expression levels of all 4 genes in vibration-treated sample groups were significantly reduced at day 10 relative to days 3 and 5. In contrast to these results, expression levels of type I collagen and elastin were relatively insensitive to vibratory stimulation. Reduced collagen and elastin expression levels were observed in samples exposed to vibratory stimulation relative to static controls at all time points, although the differences were only significant at day 5 (Figure 6.8E and F).

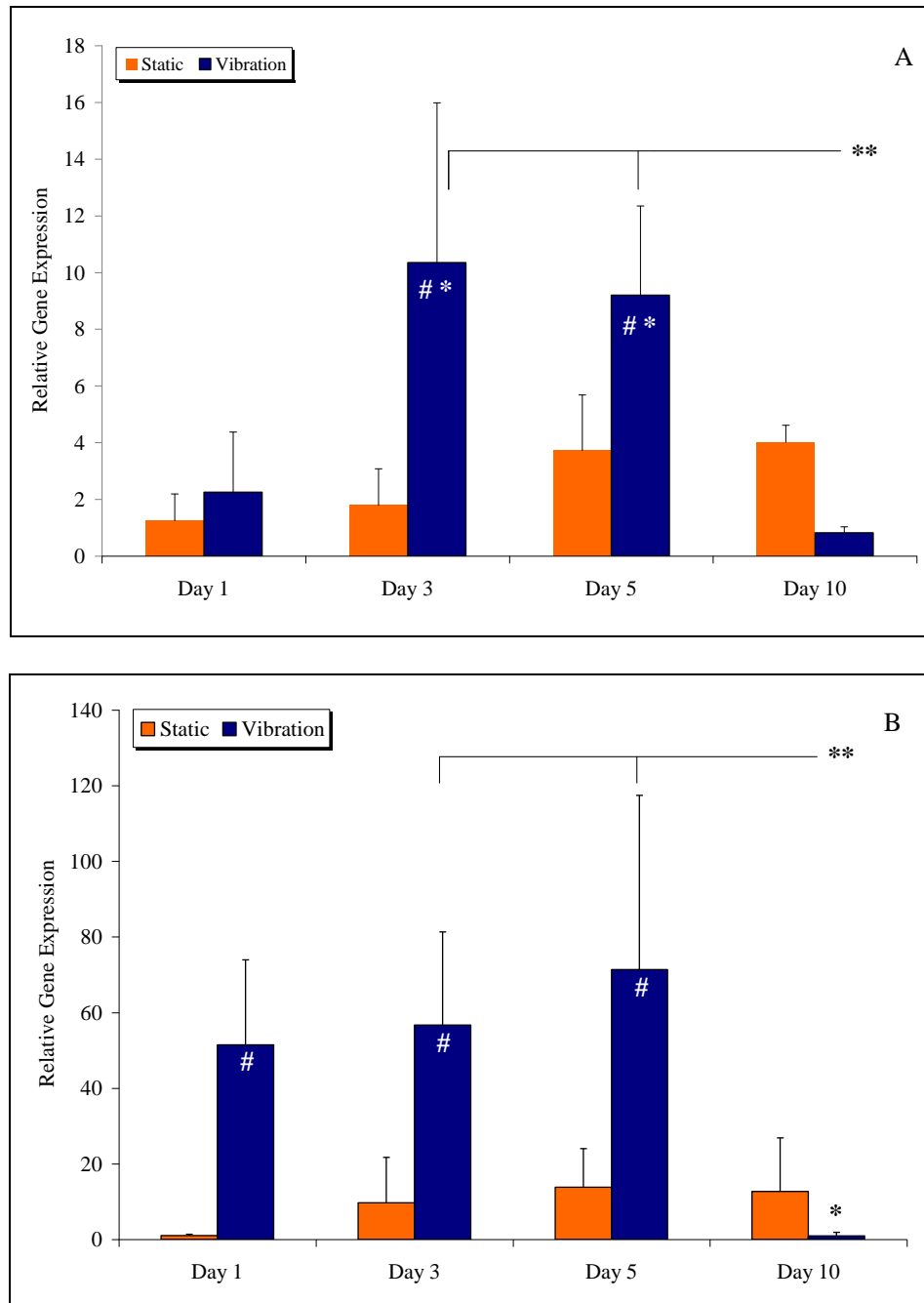


Figure 6.6 mRNA expression levels of HA Synthase 2 (A) and decorin (B). * indicates significant difference relative to day 1 of the corresponding group. # indicates significant differences relative to the corresponding static control at the same time point. ** identifies significant differences observed among other experimental groups.

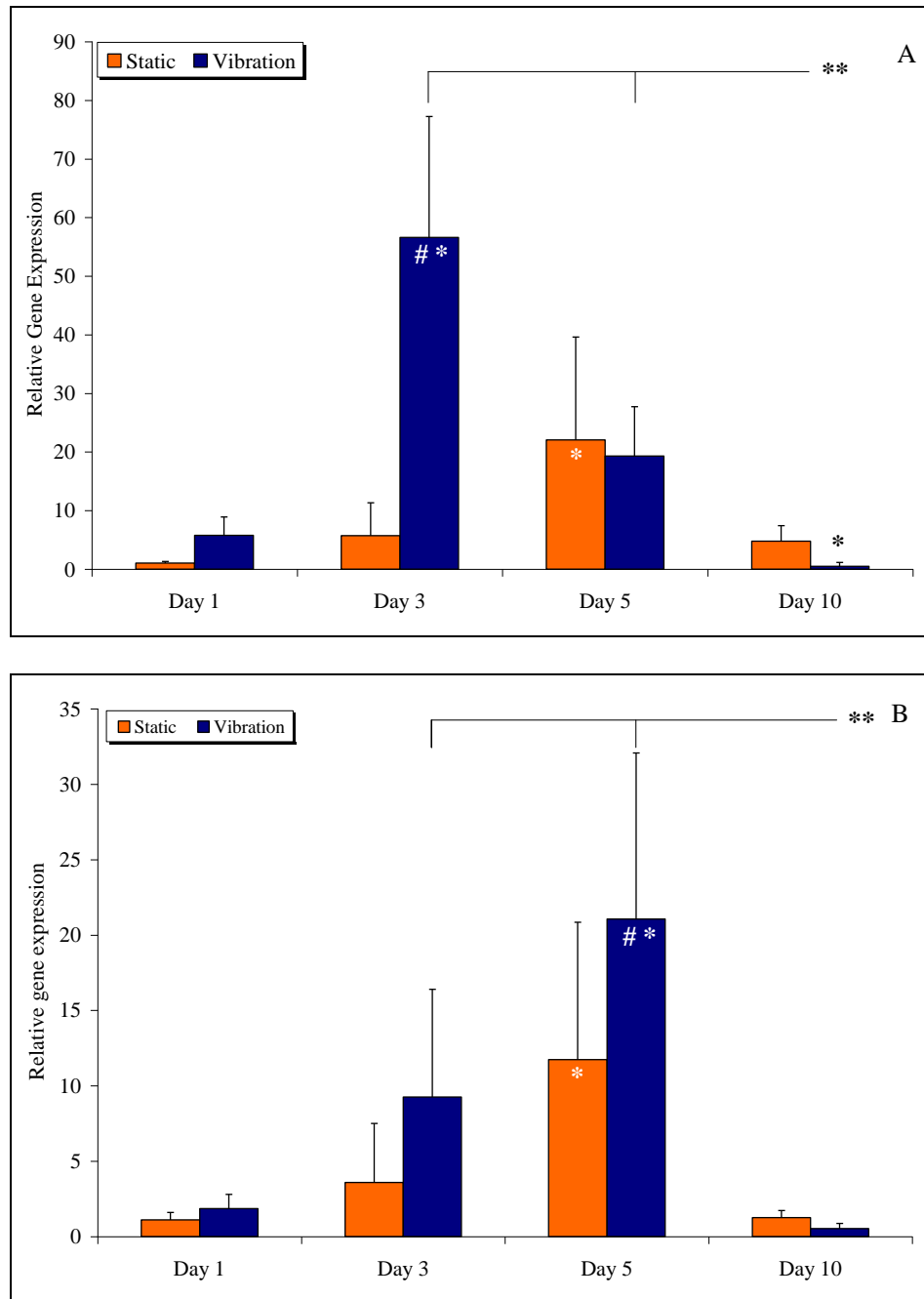


Figure 6.7 mRNA expression levels of Fibromodulin (A) and MMP-1 (B). * indicates significant difference relative to day 1 of the corresponding group. # indicates significant differences relative to the corresponding static control at the same time point. ** identifies significant differences observed among other experimental groups.

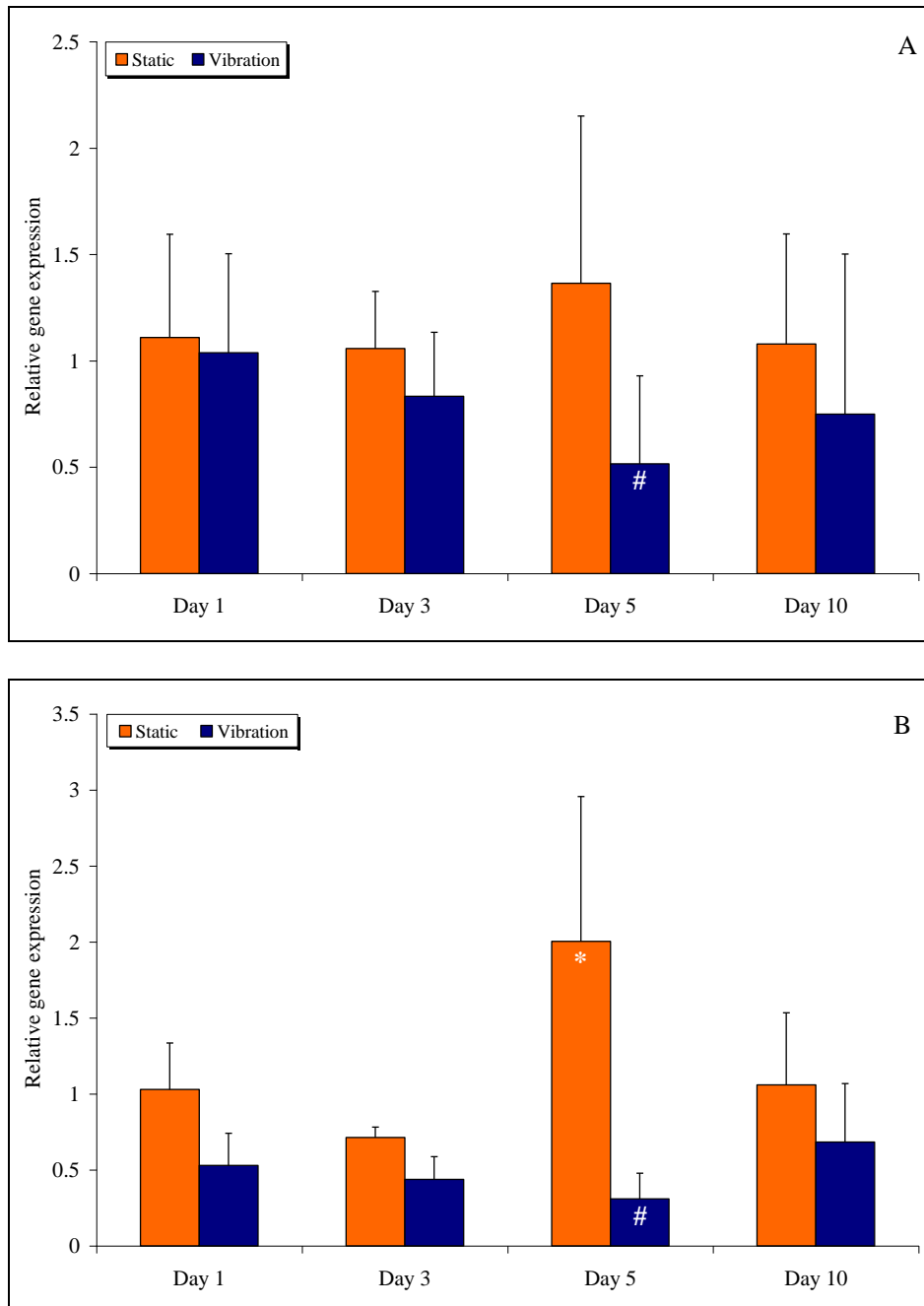


Figure 6.8 mRNA expression levels of Collagen I (A) and Elastin (B). * indicates significant difference relative to day 1 of the corresponding group. # indicates significant differences relative to the corresponding static control at the same time point. ** identifies significant differences observed among other experimental groups.

6.3.3 ECM expression

Consistent with the significant upregulation of HA Synthase 2 and proteoglycan genes at days 1, 3, and 5, the amount of proteoglycan accumulation (normalized to cell number) measured in the hydrogel constructs at days 5 and 10 was significantly higher in the vibrational groups relative to the static controls (Figure 6.9). Also, proteoglycan levels in the vibrational groups significantly increased between days 5 and 10.

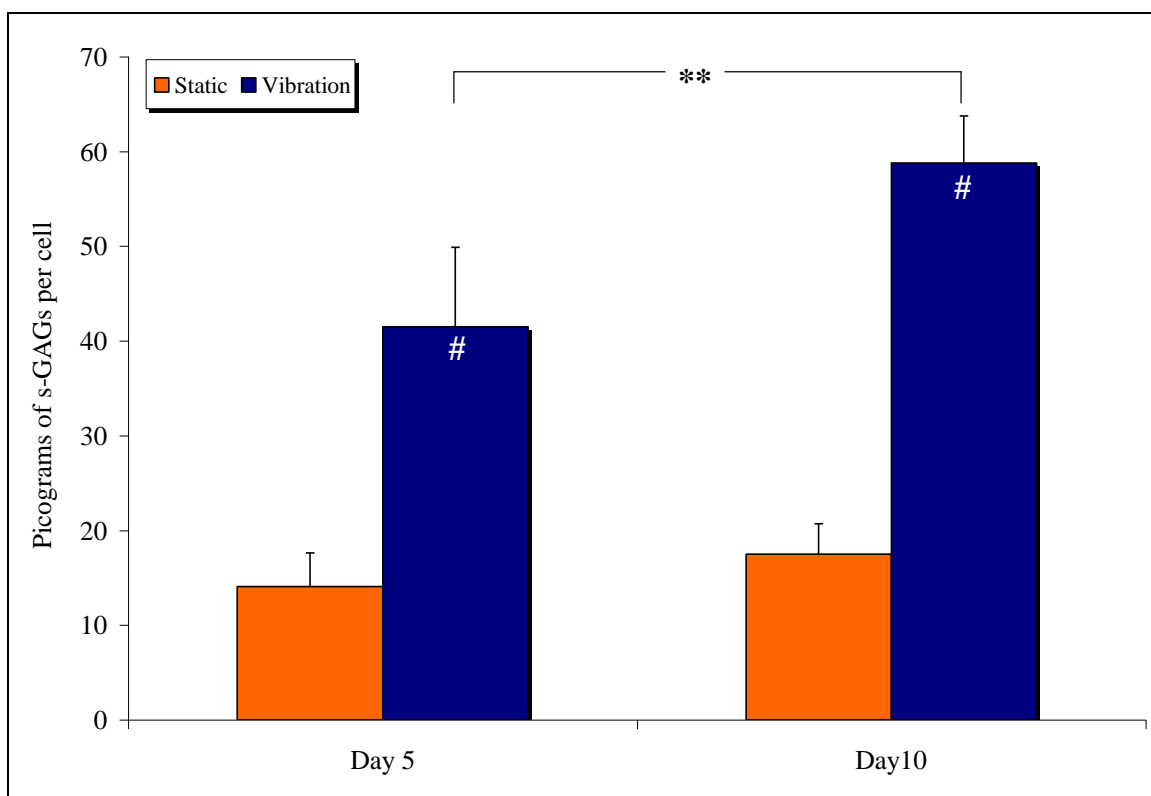


Figure 6.9 Picograms of sulfated-GAGs per encapsulated cell in the 2% w/v GMHA hydrogels after 5 and 10 days of static and vibrational culture. # indicates significant differences relative to the corresponding static control. ** indicates a significant difference among experimental groups.

Total collagen accumulation (normalized to cell number) measured in the hydrogel constructs at days 5 and 10 was significantly lower in the vibrational group relative to the static controls (Figure 6.10). In addition, total collagen levels measured in the vibrational groups significantly decreased from day 5 to day 10.

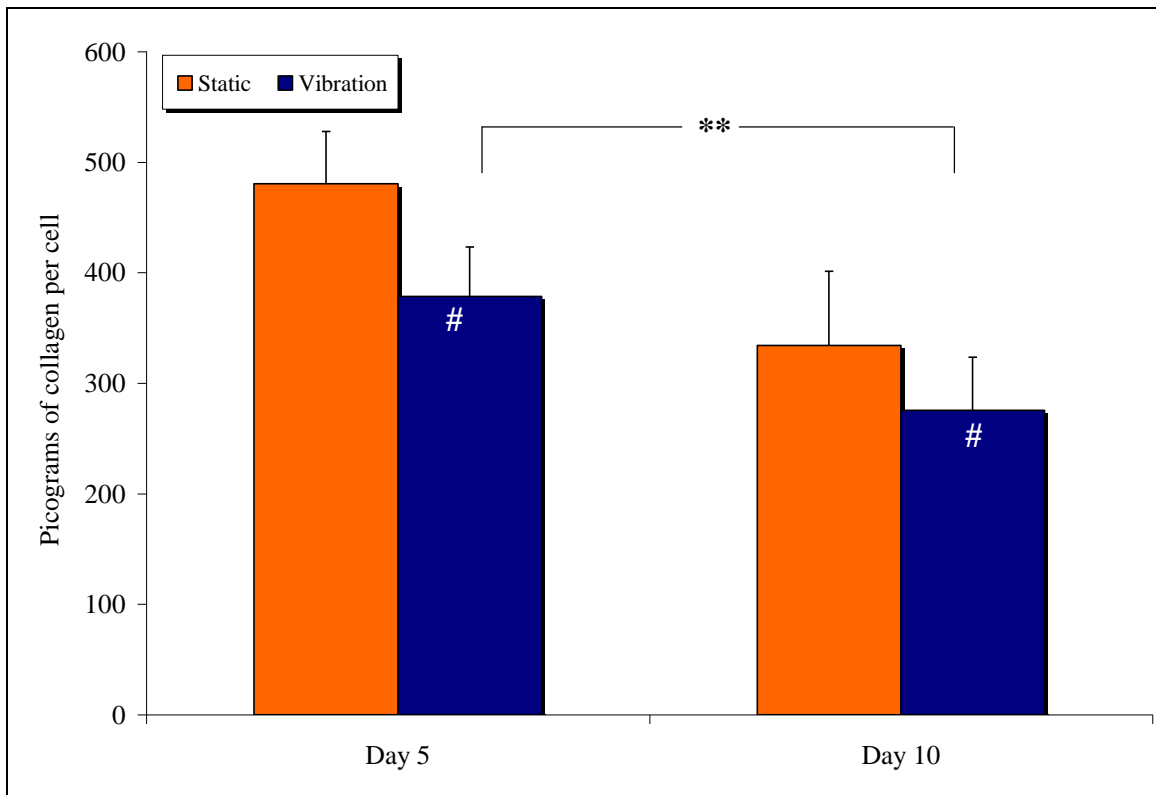


Figure 6.10 Picograms of collagen per encapsulated cell in the 2% w/v GMHA hydrogels after 5 and 10 days of culture. # indicates significant difference relative to the corresponding static control. ** indicates a significant difference among experimental groups.

6.3.4 Rheological characterization of cell-free and cell-encapsulated 2% w/v GMHA hydrogels following 10 day vibrational bioreactor culture

In order to investigate the effect of physiologically relevant high frequency vibration on the mechanical properties of the 2% w/v GMHA hydrogels, 10 day experiments were performed using cell-free and cell-encapsulated hydrogel constructs. Dynamic viscosities (Figure 6.11) and elastic shear moduli (Figure 6.12) calculated over the range of the tested frequency sweep (0.01 – 15Hz) indicated that the cell-free static controls and the corresponding vibrational samples were similar. However, the dynamic viscosities of the cell-encapsulated vibrational samples were significantly lower than the corresponding static controls and the cell-free vibrational samples (Figure 6.11). The elastic shear moduli of the cell-encapsulated vibrational samples were similar to that of the cell-free vibrational samples (Figure 6.12). Interestingly, the elastic shear moduli of the cell-encapsulated static controls were significantly higher than that of the cell-free static controls (Figure 6.12). However, when the encapsulated cell number was reduced by half, the difference in the magnitudes of dynamic viscosities (Figure 6.13) and elastic shear moduli (Figure 6.14) between the static controls and the vibrational samples became minimal over the range of frequencies tested.

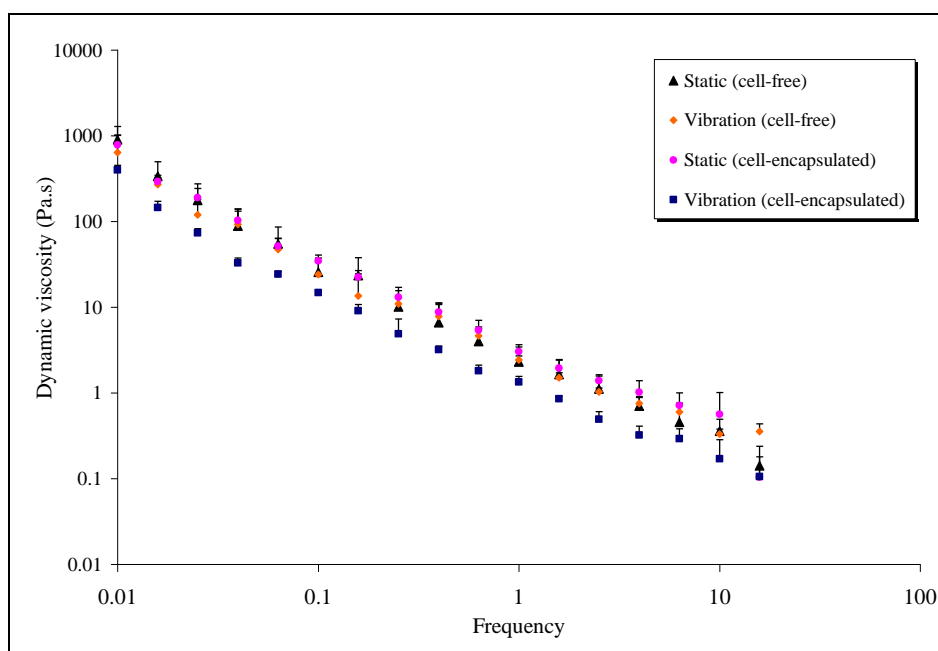


Figure 6.11 Dynamic viscosities of cell-free and cell encapsulated (4×10^6 cells/ml) 2% w/v GMHA hydrogels (static controls and vibrational samples) cultured for 10 days.

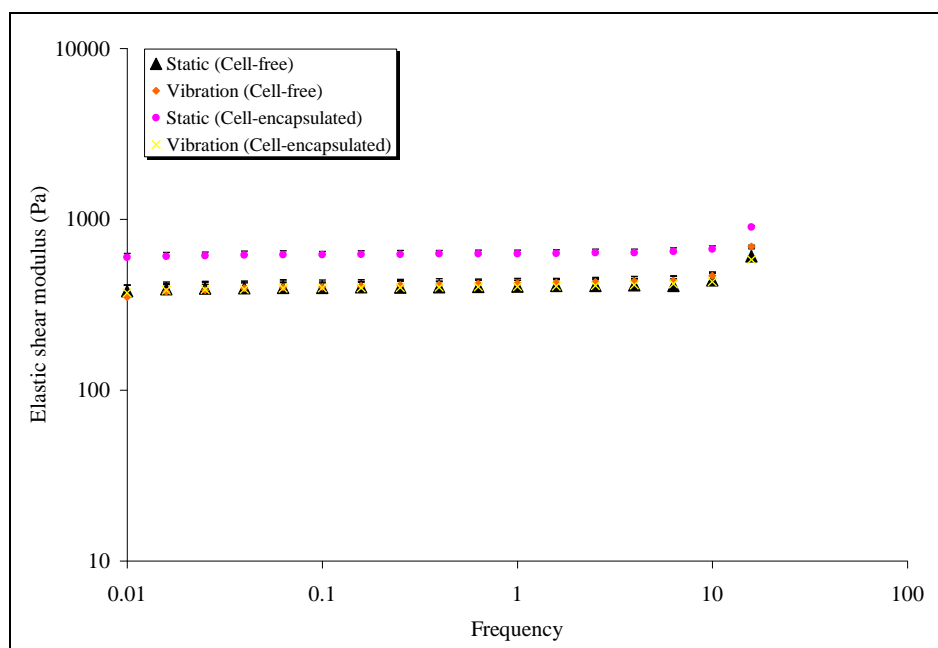


Figure 6.12 Elastic shear moduli of cell-free and cell encapsulated (4×10^6 cells/ml) 2% w/v GMHA hydrogels (static controls and vibrational samples) cultured for 10 days.

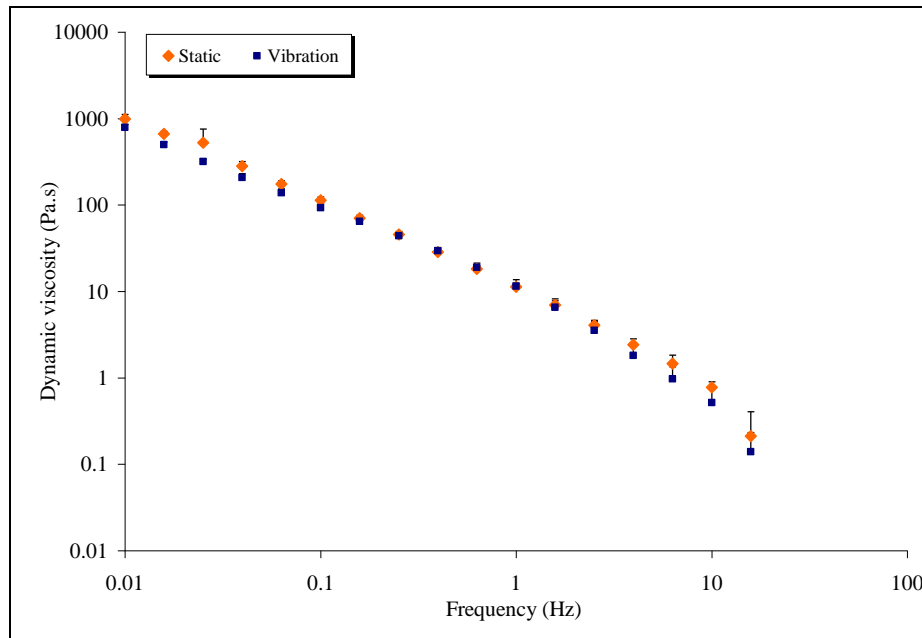


Figure 6.13 Dynamic viscosity of cell encapsulated (2×10^6 cells/ml) 2% w/v GMHA hydrogels (static controls and vibrational samples) cultured for 10 days.

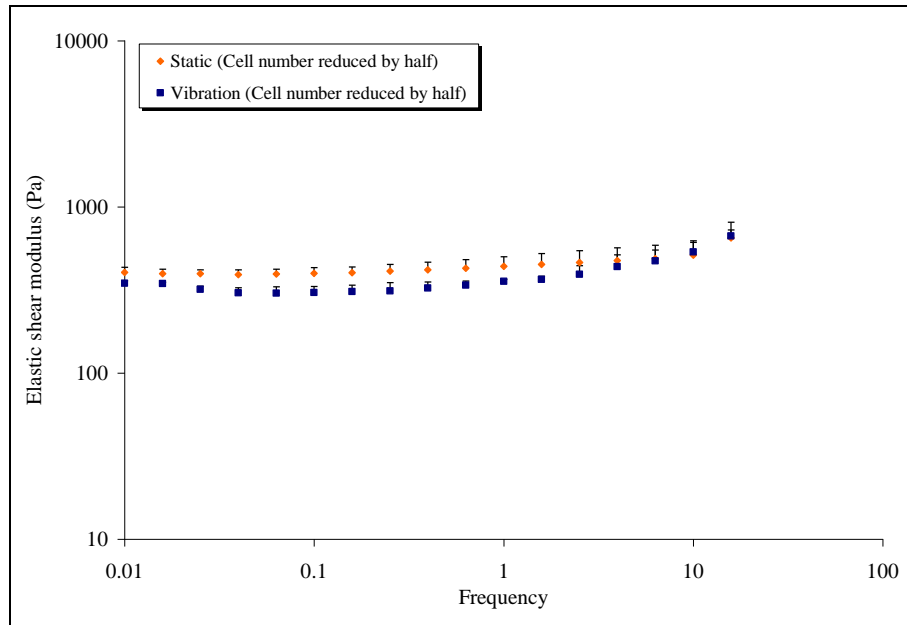


Figure 6.14 Elastic shear moduli of cell encapsulated (2×10^6 cells/ml) 2% w/v GMHA hydrogels (static controls and vibrational samples) cultured for 10 days.

6.4 Discussion

The highly differentiated organization of the vocal fold tissue helps to produce and sustain the high degrees of mechanical stresses (vibratory and cyclic strain) it is subjected to for many waking hours, daily, over an entire lifetime. Proper vocal fold tissue function being a prerequisite to normal phonation, otolaryngologists when presented with voice related problems associated with vocal fold tissue injury, must investigate the effect of the wound healing process on the native vocal fold tissue architecture/ECM composition. Previously, we have described 2% w/v GMHA hydrogels and 6% w/v PEG-based semi-IPNs aimed at restoring the architecture of the human vocal mucosa and the vocal ligament, respectively, which will facilitate long-term restoration of normal voice by preventing fibrosis during the acute stage of wound healing [297].

In the current study fibroblasts encapsulated in 2% w/v GMHA hydrogels (viscous shear properties similar to the human vocal mucosa) were subjected to physiologically relevant high frequency vibrational stimulation (100Hz sine wave, 1mm amplitude) using a vibrational bioreactor. The 2sec *on*/2 sec *off* regimen simulates a situation wherein an individual vocalizes and inhales alternately. The 2 hours/day (net vibration *on* time) mechanical stimulation regimen is selected in order to approximate the 2 hour average vocalization period for a classroom teacher in a 6 – 7hr working day [9-12]. Our results indicated that physiologically relevant, high frequency vibration alters fibroblast viability, proliferation, expression levels of some ECM related genes, and their corresponding proteins. Previously, Titze and co-workers have reported that although

6hours vibrational stimulation influenced the ECM deposition and spatial distribution of cells it did not have a significant effect on cell viability [11]. However, our results indicated that a net vibrational stimulation of 2 hours/day applied over 5 to 10 days resulted in significant reduction of cell viability [11]. The reduction in cell viability at days 5 and 10 can be attributed to the physical trauma inflicted on the encapsulated fibroblasts by the high frequency vibration over time. Also, the vibratory regimen significantly stimulated fibroblast proliferation. This is consistent with results reported previously by other researchers [11, 291, 302]. Real time RT-PCR analyses have indicated a temporal manner in the changes of mRNA expression levels of ECM related genes. Upregulated mRNA expression levels (days 1 – 5) of HA Synthase 2, decorin, fibromodulin, and MMP-1 in response to vibrational stimulation is similar to the ECM composition of the vibratory component of the human vocal mucosa. The reduction in gene expression levels at day 10 may be attributed to cellular adaptation to the vibrational regimen. High frequency vibrational stimulation has a profound effect on the expression of proteoglycans, but contrastingly it has a very mild effect on the expression of structural proteins like collagen. A significantly higher degree of proteoglycan-rich (s-GAGs) ECM deposition in the vibrational group (relative to static controls) represents an overall upregulation of ECM synthesis in response to vibrational stimulation since a decreased cell density was observed in the vibrational group (relative to the static controls) at all times points assayed.

Benign vocal fold lesions which represent the end of the wound healing cascade (nodules), or an incomplete process beyond the initial/acute inflammatory stage (polyps)

may place the vocal fold tissue at increased risk for repeated injury, leading to scarring [15]. In contrast to fetal wound healing (regenerative type), adult wound healing is characterized by scar formation attributed to decreased/degraded HA levels by day 10 [129]. Vocal fold scarring in the initial stages is associated with disorganized collagen deposition [15] and decreased amounts of HA [15], decorin [303], and fibromodulin [304]. Collagen found in scarred vocal fold tissue is reduced in density in addition to having a disorganized/compromised architecture [134]. This can be attributed to the low levels of decorin in the scarred vocal fold tissue since decorin binds collagen and regulates fibrillogenesis [15]. It has been reported previously that HA levels in the vocal fold wounds decrease to subnormal levels after day 5 of injury resulting in vocal fold tissue scarring [131]. However, chronic vocal folds scars (2 to 6 months post injury) reveal HA levels similar to that of normal tissue [131-134]. Scarring brought about by a change in the ECM composition of the vocal mucosa during the acute phase of wound healing alters vibratory biomechanics of the tissue which ultimately results in dysphonia. Vocal fold scarring and associated pathologies are the most challenging voice related problems otolaryngologists are faced with today [18]. Apart from voice therapy, treatment of vocal fold scarring involves surgical techniques which rely mainly on implanting natural/synthetic biomaterials to improve vocal mucosa pliability and alleviate glottic incompetence. However, none of the existing treatment techniques achieve long-term success due to their inherent inability to restore the native tissue architecture/ECM composition.

Regenerative-type repair in fetal wounds is attributed to sustained high levels of HA throughout the wound healing phase [128, 130, 269]. Our results indicated that in addition to approximation of the viscous shear properties of the human vocal mucosa, the 2% w/v GMHA hydrogel formulation may serve to sustain elevated levels of HA and proteoglycans at the site of injection during the acute phase of wound healing. Also, exogenous HA is reported to decrease collagen synthesis and deposition in the ECM without affecting de novo HA synthesis [127]. Our results indicated a significant reduction in total collagen synthesized in the vibrational samples after 5 and 10 days of culture. The upregulation of mRNA levels of HA Synthase 2, decorin, and fibromodulin and the corresponding proteins have an effect on collagen fibrillogenesis [127] as indicated by our results. It is logical that increased proteoglycan synthesis and decreased collagen synthesis could play a vital role in averting scar formation. Thereby, a significant increase in the proteoglycan content and a decrease in the collagen content of the vibrational samples relative to the static controls is an indication that the 2% w/v GMHA hydrogel formulations may prevent scarring and facilitate vocal mucosa specific ECM deposition under vibrational stimulation in vivo.

Rheological analyses did not reveal any significant differences in the magnitudes of dynamic viscosities and elastic shear moduli between the vibrational samples and the static controls in the cell-free group over the range of frequencies tested. Thereby, one may infer that the vibrational regimen had minimal effect on the crosslinking density of the GMHA hydrogels. We hypothesize that reduction in the dynamic viscosities of the cell-encapsulated vibrational samples (relative to corresponding static controls) despite

significant upregulation of proteoglycan synthesis per cell, can be attributed to accelerated cellular remodeling of the hydrogel scaffold and faster ECM turnover rate over a 10 day time course under vibration. During the eventual in vivo application, the neo-matrix synthesized by the transplanted/endogenous cells, will be held in place more efficiently due to bonding with the surrounding tissue. Interestingly, the rate of cellular remodeling of the GMHA hydrogel matrix was reduced by encapsulating half the original cell number, as a result of which the difference in the dynamic viscosities and elastic shear moduli between the vibrational samples and the static controls was minimal. Higher magnitudes of elastic shear moduli recorded for the static controls relative to corresponding vibrational samples (original cell number) may be due to significant increases in the cell density and the amount of collagen synthesized per encapsulated cell in the static controls.

Due to distinct biomechanical properties of the human vocal mucosa and approximation of these properties by the 2% w/v GMHA hydrogel formulation, use of these hydrogels was aimed at restoring mechanical integrity and vocal mucosa function following injury [297]. Our results suggested that restoration of the vibratory microenvironment using the 2% w/v GMHA hydrogels, will facilitate regeneration of the native human vocal mucosa ECM matrix composition and thereby normal phonatory behavior. We are cognizant of the importance of the biological environment within the human body in terms of the occurrence of inflammation and its effect on scaffold degradation post implantation. A classic case of inflammation may lead to minimal cellular infiltration/colonization and a thick collagen based capsule isolating the scaffold.

We hypothesize that this kind of adverse inflammatory reaction may not occur in response to vocal fold tissue augmentation using GMHA hydrogels since the vocal fold lamina propria is minimally vascular.

6.5 Conclusion

Through the use of a vibrational bioreactor to condition fibroblast differentiation, we have shown that the 2% w/v GMHA hydrogel encapsulated-cell formulation may help restore native vocal fold tissue (vocal mucosa) architecture following injury and thereby restore long-term normal phonatory voice. Functional vocal mucosa regeneration following injury may provide a rational basis for the structural/functional design of engineering tissues for an eventual clinical application.

CHAPTER 7

CONCLUSION AND RECOMMENDATIONS

7.1 Conclusion

The inability of current treatment techniques to restore long-term normal phonatory voice following scarring related vocal pathology, has given rise to the need for the “Vocal Fold Regeneration Project” in the Micro-Environmental Engineering Laboratory (μEEL). This project is based on the hypothesis that rapid restoration of the mechanical microenvironment using mechano-mimetic scaffolds will facilitate vocal fold tissue-specific ECM production and ultimately restore normal phonation by preventing scarring sequelae. Towards this end, we created *in situ* photopolymerizable, degradable, cytocompatible PEG-based semi-IPNs and GMHA hydrogels that approximated the elastic (vocal ligament) and rheological (vocal mucosa) properties of the highly differentiated human vocal fold tissue.

During the course of our preliminary experiments and literature review we realized that the nanometer-scale mesh size of the PEG-based hydrogel networks restricts encapsulated cells to a rounded morphology that can inhibit cellular processes such as proliferation, migration, and ECM production that are essential for the early stages of remodeling and tissue formation. Therefore, the first phase of the vocal fold regeneration project was dedicated to investigating an approach for accelerating cellular remodeling based on the creation of semi-IPNs composed of hydrolytically degradable PEG diacrylate macromers and native, enzymatically degradable ECM components (collagen,

gelatin and HA. Among the three ECM components investigated, addition of HA at concentrations of 0.12% w/v and greater supported fibroblast spreading throughout the 3-dimensional network and significantly increased proliferation relative to control hydrogels without HA. Incorporation of HA resulted in relatively small changes in hydrogel physical/chemical properties such as swelling, degradation rate, and elastic modulus. Fibroblast spreading was eliminated by the addition of Hase inhibitors, demonstrating that cell-mediated enzymatic degradation of HA is a necessary mechanism responsible for the observed increases in fibroblast activity. **By accelerating early cellular remodeling and growth, these PEG-based semi-IPNs may be useful vehicles for cell transplantation in a variety of tissue engineering applications in addition to vocal fold tissue repair.**

The objective of the second phase of this project was the development of mechano-mimetic, degradable hydrogels that may be photopolymerized *in situ* following vocal fold injury or surgery using minimally invasive techniques to fill vocal fold defects. **PEG diacrylate based semi-IPNs (6% w/v) and GMHA hydrogels (2% w/v) were found to provide elastic and rheological properties consistent with the human vocal ligament and the vocal mucosa, respectively.** Cell culture studies indicated that these semi-IPN/hydrogel materials supported cell spreading, cell proliferation, and ECM deposition throughout the 3-dimensional crosslinked network.

The third phase of this dissertation investigated the hypothesis that rapid restoration of the vibratory microenvironment will facilitate vocal mucosa-specific ECM production, and ultimately restore normal phonation. Towards this end, we assessed the

ability of the 2% w/v GMHA hydrogels to support human fibroblast formation of vocal mucosa-like ECM in response to physiologically relevant high frequency vibration using a custom-made vibrational bioreactor. **Exposure of GMHA hydrogel-encapsulated fibroblasts to physiologically relevant high frequency vibration stimulated a pattern of ECM expression (upregulation of GAGs, downregulation of fibrous matrix proteins) consistent with the composition of the native human vocal mucosa.** Studies analyzing vibration-induced changes in hydrogel mechanical properties and quantitative analysis of proteoglycans and collagen synthesis following long-term vibrational bioreactor studies indicated that these GMHA hydrogels introduced into the human vocal mucosa during the acute phase of wound healing by minimally invasive methods may bring about regeneration of the native vocal mucosa-ECM composition, and thereby restore normal phonation.

In the broad context of tissue engineering, the data presented in this dissertation indicates that GMHA- and PEG-based hydrogel/semi-IPN encapsulated fibroblasts may be useful, site-specific, autologous cell delivery matrices for a variety of soft tissue repair/regeneration applications. The ability to tailor mechanical properties/degradation rates to suit the eventual application, which derives from the vast range of macromers (and possible blends) synthesized, in addition to metabolizable degradation products, lends versatility to these matrices. Although several groups have investigated the effect of cyclic strain/compression on cell behavior, few have investigated the effect of physiologically relevant high frequency vibration on cellular function. Through our vibrational culture studies over a 10 day time course, we have significantly contributed to

the understanding of the effect of physiologically relevant high frequency vibration on the gene/protein expression of cells cultured in 3-dimensional crosslinked substrates.

7.2 Limitations

These GMHA / PEG diacrylate-based hydrogels / semi-IPNs are not intended to soften existing mature scar tissue. These materials are intended to fill critical size defects following some kind of injury or surgery in an attempt to reintroduce the vibrational stimulus that may help restoration of the normal tissue matrix composition. In case of mature scars, the scar tissue can be excised followed by use of these GMHA / PEG-based hydrogels / semi-IPNs to fill the resulting defect.

Few existing biomaterials can replicate the complex, nonlinear stress-strain response of the human vocal ligament. For this application, PEG hydrogels were chosen based on their well-documented biocompatibility and elastic mechanical properties, which are consistent with the linear stress-strain properties of the vocal ligament at low strain levels (0-15%). The mechanical properties of hydrogels in the swollen state are primarily a function of the crosslinking density, which may be controlled through variation of polymer molecular weight, concentration, and degree of functionalization [305, 306]. In this study, the crosslinking density and subsequent tensile properties of PEG-diacrylate hydrogels were manipulated through variation of macromer concentration. At 6% w/v concentration, PEG hydrogels closely approximated the linear stress-strain response and elastic modulus reported for the human vocal ligament at low strains [63]. Although strain to failure approached physiological levels (Figure 5.6), the

nonlinear stress-strain response observed in native tissue between approximately 15 and 45% strain was not achieved. These results are consistent with the rubberlike behavior of such highly crosslinked polymer networks, which exhibit viscoelastic behavior only at very low temperatures [306]. The in vivo functional efficacy of these materials will be dependent on physiological strain levels experienced during phonation. To our knowledge, no studies have obtained direct, empirical measurements of vocal ligament strains during phonation. Previous reports in the literature have described physiological strain levels as ranging from 0 – 15% [8] to as high as 45 – 60% [277, 307]. Titze et. al. recently reported a mathematical model of vocal ligament vibration that extended conventional vibrating string models to include bending stiffness and variable cross section; this model predicted higher frequencies (1000 Hz) can be attained at lower strains (50%) than previously expected [308]. Based on this model, it is possible that at typical phonation frequencies (100 – 300 Hz) [12], ligament strains may remain close to the 15% range in which PEG hydrogels closely approximate the native tissue properties.

In the present study, elastic shear modulus and dynamic viscosity of hydrogels crosslinked from methacrylated HA varied as a function of macromer concentration. Due to HA's high molecular weight, these results are likely to be attributable to concentration-dependent effects on both the covalent crosslinking density and polymer chain entanglement [101]. GMHA hydrogels crosslinked from 1.5 – 2.5% solutions exhibited dynamic viscosities approximating the range of values reported for native human vocal mucosa [103, 195, 288], which was highly variable due to intersubject differences attributed to age and gender [103]. However, the elastic shear moduli values were

several fold higher than the average values reported for human vocal mucosa [276]. These results, consistent with other reports describing rheological analysis of chemically crosslinked HA hydrogels [43, 200], suggest that the degree of covalent crosslinking in these materials may exceed the molecular-bonding interactions in native tissue, resulting in increased stiffness. Elastic modulus values above the physiological range may in fact be advantageous for in vivo applications. Enzymatic degradation of naturally derived biomaterials during the acute inflammatory phase following implantation is likely to produce a transient decrease in mechanical properties before effective synthesis and organization of new matrix by colonizing or transplanted cells. A limitation of our rheological testing is that data could not be obtained at physiologically-relevant frequencies. Several studies have recently described equipment and methods for performing accurate rheological analysis at physiological frequencies [200, 309, 310].

Our rheological studies indicated approximately an order of magnitude difference between the dynamic viscosities of the 2% w/v unmodified HA solution and the 2% w/v GMHA solution at all frequencies tested. Since HA viscosity is largely a function of molecular weight at fixed concentration [311], this observation probably indicates that some degree of HA degradation and reduction in molecular weight occurred during the methacrylation and purification processes. Although partial degradation could pose a challenge to reproducibility, swelling and rheological properties of hydrogels prepared from various batches of GMHA were highly consistent (data not shown). While at least two alternative synthetic routes using methacrylic anhydride and N-3-aminopropyl methacrylamide have been described [281, 312], the effect of these procedures on HA

molecular weight or rheological properties relative to the native polymer have not been reported.

Normal human dermal fibroblasts (NHDF) were used in these studies as a model cell type. Due to its limited intrinsic cellularity, cell transplantation may be important for efficient restoration of vocal fold ECM. Limited cellularity combined with potential scarring complications following biopsy of healthy autologous tissue suggests that vocal fold fibroblasts may not be a feasible cell source for transplantation. In contrast, dermal fibroblasts are readily available in vast quantities. To our knowledge, no study at present has fully characterized the phenotypic differences between fibroblasts derived from the vocal folds relative to other tissues, or identified an optimal cell source for vocal fold transplantation. We use these cells to demonstrate that our materials support cell proliferation and matrix synthesis, but do not claim that these cells behave identically to vocal fold fibroblasts.

7.3 Recommendations

Based on observations and data analyses from our research till date, I would like to make the following recommendations aimed at furthering the scope of our research efforts.

7.3.1 Specific aims

Aim 1: To characterize the downregulation of newly synthesized collagen by the GMHA hydrogel encapsulated cells in response to high frequency vibration in

terms of the architecture/spatial organization. Also, investigate the mRNA expression levels of TGF- β 1, TGF- β 1 receptor, hyal-1, hyal-2, lysyl oxidase, and HGF. This is vital if we are to conclusively prove that restoration of the vibratory microenvironment using the GMHA hydrogels immediately following injury can prevent fibrotic scarring.

Aim 2: To investigate the effect of physiologically relevant cyclic strain on :-
(a) the ECM synthesis of 6% w/v PEG-based semi-IPN encapsulated fibroblasts and
(b) the mechanical properties of these PEG-based matrices following long-term bioreactor studies.

Aim 3: To model vocal overuse using the vibrational bioreactor and to investigate the gene/ECM expression of GMHA hydrogel encapsulated fibroblasts in response to the extended mechanical loading over a time course.

Aim 4: To investigate the hypothesis that application of localized high frequency vibration is a potential anti-fibrotic treatment. Briefly, high frequency vibration applied periodically to fresh wounds through carbon nanotubes (using DNA or electrolytes as a power source) encapsulated in GMHA/PEG based hydrogels may :- (a) stimulate infiltrating fibroblasts to produce native tissue-like ECM and (b) alter growth factor profiles in the wound which may help prevent fibrotic scarring.

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