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Therapeutic potential of gel-based injectables for vocal fold regeneration

Rebecca S. Bartlett, MA^{*}, Susan L. Thibeault, PhD^{*}, and Glenn D. Prestwich, PhD[§]

^{*}5107 Wisconsin Institutes for Medical Research, 1111 Highland Avenue, Division of Otolaryngology – Head and Neck Surgery, University of Wisconsin – Madison, Madison, Wisconsin, United States

[§]Department of Medicinal Chemistry and Center for Therapeutic Biomaterials, University of Utah, 419 Wakara Way, Suite 205, Salt Lake City, UT 84108, Salt Lake City, Utah, United States

Abstract

Vocal folds are anatomically and biomechanically unique, thus complicating the design and implementation of tissue engineering strategies for repair and regeneration. Integration of an enhanced understanding of tissue biomechanics, wound healing dynamics and innovative gel-based therapeutics has generated enthusiasm for the notion that an efficacious treatment for vocal fold scarring could be clinically attainable within several years. Fibroblast phenotype and gene expression are mediated by the three-dimensional mechanical and chemical microenvironment at an injury site. Thus, therapeutic approaches need to coordinate spatial and temporal aspects of the wound healing response in an injured vocal tissue to achieve an optimal clinical outcome. Successful gel-based injectables for vocal fold scarring will require a keen understanding of how the native inflammatory response sets into motion the later extracellular matrix remodeling, which in turn will determine the ultimate biomechanical properties of the tissue. We present an overview of the challenges associated with this translation as well as the proposed gel-based injectable solutions.

I. Introduction

Vocal fold scarring is the result of trauma, surgery to remove benign or malignant lesions, prolonged endotracheal intubation, radiation, or burns, and can have a deleterious effect on vocal quality. While there is a paucity of data about the specific impact of vocal fold scarring on quality of life, it is known that more than half of dysphonic individuals attribute their dysphonia as having a negative impact on their social interactions, career and mental health [1]. To date, traditional surgical and behavioral treatments have yielded inconsistent results [2, 3], marking vocal fold scarring as a recalcitrant clinical issue. Over the last fifty years, the introduction of vocal fold injectables to treat the dysphonia that results from vocal fold scarring has been met with limited success. Enhanced understanding of vocal fold anatomical and biomechanical properties has driven the technological advancements in hydrogels.

2. Background

The larynx is a valve which sits at the fork of the respiratory and digestive tracts. The paired vocal folds are housed within the larynx and are just superficial to the airway. During infancy, the larynx is positioned high in the throat which effectively separates the

swallowing passageway from the airway. This orientation is biologically useful for infants, as it minimizes the risk of fluids being aspirated into the airway. During human development, the larynx gradually descends which results in communication between the respiratory and digestive tracts (e.g., which can be realized when one chokes on food or fluid), but allows for greater independent control of phonatory and articulatory manipulations during speech [4]. During speech, humans have a sophisticated capacity for modulating vocal frequency (i.e., pitch), intensity (i.e., loudness) and vibratory mode (i.e., register) by making laryngeal and supralaryngeal adjustments.

The vocal folds are composed (from superficial to deep) of epithelium, superficial lamina propria (SLP), intermediate lamina propria, deep lamina propria (DLP) and the thyroarytenoid muscle (see Figure 1). During phonation, the vocal fold cover (epithelium and the SLP) comprise the vibratory unit overlying the largely stationary vocal fold body (DLP and muscle). Vocal fold dynamics occurs in three dimensions. A lateral to medial movement of the entire vocal fold allows for glottic opening (i.e., for breathing) and glottic closing (i.e., for phonation and airway protection). Anterior to posterior manipulations of the vocal fold caused by activation of the cricothyroid muscle cause the tissue to lengthen (i.e., increasing pitch) or shorten (i.e., decreasing pitch). An inferior to superior vertical shear wave of the vocal fold cover/mucosa occurs when the vocal folds are adducted and there is ample subglottic air pressure to induce phonation. This small amplitude, high frequency vibration (100–1000 Hz) of the vocal fold mucosa is the sound source during phonation [5].

When vocal fold vibration is disrupted due to altered biomechanical properties, increased mass, irregularly shaped vocal fold edge or impaired neuromuscular control, dysphonia (i.e., voice impairment) can occur. Incomplete vocal fold closure defines glottal incompetence and results in air escape during vibration. This can impart a “hoarse” or “breathy” quality to the voice. Injectables have been delivered into the thyroarytenoid muscle or the vocal fold lamina propria depending on the underlying vocal fold pathology. Muscle-directed treatment is intended to increase tissue bulk, encourage medialization, and improve glottic closure in patients with vocal fold paralysis/paresis or presbyphonia. There are a number of injectable biomaterials which have been reported to demonstrate consistent success in these populations and therefore will not be a focus of this paper. Muscle-directed biomaterials do not require the biomechanical sophistication of those that are directed at the vocal fold mucosa.

Lamina propria-directed injectables are used to treat the disrupted viscoelastic properties and irregular vocal fold edge associated with vocal fold scarring and/or sulcus. Limited and inconsistent success with treating these populations has inspired significant innovation of biomaterials for attenuating scar. Lamina propria scarring is marked by an alteration of the composition and arrangement of extracellular matrix (ECM) proteins; these elements directly determine the viscous and elastic properties of the tissue. There is no “gold standard” of treatment for vocal fold scarring at this time.

Otolaryngologists and speech pathologists have employed a variety of treatments for vocal fold scarring which have been met with inconsistent results. Microsurgical treatment has included scar lysis/excision which is targeted at releasing tissue fibrosis, medialization laryngoplasty which is intended to improve glottal closure, and engraftment of tissue flaps/grafts which is meant to implant unscarred tissue [2]. The graft could act as a separation layer between the mucosa and ligament to prevent pathological re-tethering of the layered vocal fold tissue. Further, bioimplants of various biomaterials such as collagen and fat have been used to fill space and encourage some biological activity. It should be noted that surgical management (not including injection) carries a risk of additional iatrogenic fibrosis. Behavioral therapy (i.e., voice therapy) can be offered to balance the respiratory, phonatory

and resonance subsystems to optimize vocal quality and reduce vocal effort/fatigue. Providing education about vocal hygiene may also assist the patient in management of secondary contributing factors (reflux, hydration, excessive voice use, etc).

Over the past fifteen years, biochemically advanced injectable hydrogels have been investigated which have the capacity to modulate the inflammatory response and direct extracellular matrix remodeling [6, 7] (see Table 1). Injectable hydrogels may be an ideal treatment method for vocal fold scarring for four distinct reasons. First, as compared to surgical implantation and scar lysis procedures, delivery of the hydrogel by injection minimizes the degree of mucosal violation and the invasiveness of the procedure. A preventive delivery approach to minimize ECM injury and loss is essential. The lamina propria of the vocal folds is only 2–3 mm thick [8], so the possibility of impairing the mucosal wave is present with every microsurgical procedure. Second, hydrogel injection can be performed as an in-office procedure under local anesthetic. This enhances the accessibility of treatment of vocal fold lamina propria surgery for a greater number of patients and precludes the risk associated with general anesthesia. Third, injectable hydrogels can be designed to crosslink *in situ*, which offers the unique advantage of being able to conform to the complex vocal fold dimensions once delivered. The crosslinkable polymer mixture would adhere to the tissue during gel formation and the resulting mechanical interlocking that would arise from surface microroughness would strengthen the tissue-hydrogel interface and could minimize biomechanical interruption during vibration. Lastly, cells can easily be encapsulated within injectable hydrogels which are designed to crosslink *in situ*, and as such be delivered as part of a combination therapeutic. The cells must be present prior to crosslinking in order to be incorporated into the scaffold. As the cells are present during gelation, cytocompatible chemistries and materials must be used. It should be noted that currently most hydrogels intended for use in the vocal fold are already crosslinked at the time of purchase, but there are emerging biomaterials which are designed to crosslink *in situ* which could enhance clinical possibilities.

3. Therapeutic Considerations and Challenges

There are three main considerations specific to the therapeutic response of vocal folds which must be addressed when designing and evaluating gel-based injectables: (i) inflammatory response, (ii) ECM remodeling response, and (iii) biomechanical response. The following discussion will mirror the *in vivo* chronology of these considerations.

3.1 Inflammatory Response

3.1.1. Normal physiology and pathophysiology—Wound healing is an intricately coordinated and elegant process. It is marked by an initial inflammatory phase which is critical for initiating cytochemical interactions which have downstream effects. Comprehensive evaluation of the inflammatory response invoked by a biomaterial injected into the vocal folds is critical due to their location. As the vocal folds are a valve at the top of the airway, a strong inflammatory response marked by edematous swelling could fatally restrict the airway. Gel-based injectables which have been evaluated thus far have reported excellent safety profiles within this regard. The early, mild inflammatory responses that have been reported have demonstrated biological utility for initiating the wound healing process rather than posing a biological risk [9–11].

The acute inflammatory phase of wound healing persists from the time that the injury occurs to approximately four to six days post-injury [12]. Figure 2 is a histological image of vocal fold tissue three days following surgical injury. The onset of this phase is marked by the body controlling the bleeding via vasoconstriction of the affected vessels. In the vocal fold lamina propria there are only small vessels (i.e., capillaries, arterioles and venules). They

have anterior and posterior origins and are oriented parallel to the vocal fold edge [13]. Capillaries from the left and right vocal folds anastomose at the anterior commissure (the apex where the vocal folds converge) [14]. As visualized by scanning electron microscopy, there is little to no vascular communication between the lamina propria and the thyroarytenoid muscle [14]; this may contribute mechanical freedom during vibration. The vasculature in the superficial lamina propria is sparse, and increases in density with greater depth of the vocal fold [13]. The vocal fold mucosa is significantly less vascularized than the thyroarytenoid muscle, which may offer an advantage for preventing phonotraumatic hemorrhage in the lamina propria. Regardless, when scar-inducing injury to the vocal fold lamina propria occurs and the microvasculature is violated, local hemostasis provides the foundation for future wound healing.

Platelets from the circulating blood supply aggregate at the injury site and activate a coagulation response. The clot becomes the headquarters for inflammatory cells (i.e., neutrophils, monocytes) and keratinocytes, as well as cells which will be important for the later stages of matrix remodeling (i.e., fibroblasts). Neutrophils and monocytes are attracted to the wound bed through a variety of chemotactic signals released by the platelets and the clot (e.g., interleukin-1: IL-1, tumor necrosis factor-alpha: TNF- α transforming growth factor beta-1: TGF- β 1) [12]. Neutrophils bind to the pre-existing ECM by surface integrins and are important for cleaning bacterial agents from the injured area [12]. They also release matrix metalloproteinases (MMPs) that are able to break up the surrounding ECM. Further, it has been shown that neutrophils are a source of pro-inflammatory cytokines which are involved with downstream recruitment of fibroblasts [15]. Monocytes differentiate into macrophages 48–96 hours after injury and are involved in phagocytosis of apoptotic neutrophils, debris and bacteria at the wound bed. They also release a variety of growth factors and cytokines (i.e., platelet derived growth factor: PDGF, TNF- α interleukin-6) in order to encourage migration of more macrophages and fibroblasts [12, 16]. Keratinocytes, monocytes, macrophages and fibroblasts express MMPs when exposed to TNF- α which are able to further break up the damaged ECM at the injury site.

3.1.2. Role of hydrogels in inflammation and healing—When considering injectable hydrogel immunocompatibility for a clinical population, two major potential sources of inflammation are the biomaterial composition (i.e., chemistry and surface characteristics) as well as surgical trauma [11]. Prophylactic application of the hydrogel could amplify the inflammatory response at the surgical site, whereas hydrogel treatment applied to an established vocal fold scar could result in a replication of the inflammatory response. In both situations, there could be damaging downstream consequences for the wound bed.

One can envisage two main types of injectable hydrogels, which have been classified as “monolithic” or “living” [17]. Monolithic hydrogels have fixed chemistry, do not change physical form, have tightly linked biomechanics and degradation times, and are poor vehicles for cell therapy. In contrast, living hydrogels offer the opportunities to vary chemistry, modify physical forms, separately control biomechanics and degradation times, and introduce cells in suspension [18].

The monolithic hyaluronic acid (HA) derived hydrogel Hylaform® (Hylan-B gel) has been reported to be non-inflammatory in animal models based on lack of breathing difficulties and histological analysis lacking the appearance of macrophages and other inflammatory cells at 1, 3, 6, and 12 months post injection [19, 20]. Hylan-B is a divinylsulfone-crosslinked derivative of HA. In a clinical evaluation of Hylan-B gel injection in five patients with vocal fold scarring, no immediate or later edema, airway obstruction or bleeding were observed [21]. A similar result was observed in a rabbit model with a mild

response at 1 week, resolving by 3 weeks, using Restylane®, a monolithic butane-1,4-diol diglycidyl ether-crosslinked derivative of HA [22].

Recently, a clinically useful living HA hydrogel has been developed for wound repair, adhesion prevention, and cell therapy applications [18, 23]. This hydrogel is based on a carboxymethylated derivative of HA that was chemically modified to incorporate thiol residues. The thiol groups can be chemically crosslinked within minutes under mild, physiological conditions, a characteristic important for the incorporation of cells or active molecules into an injectable biomaterial. Previously termed Carbylan-S, for carboxymethyl hyaluronan, this material is now available for research use as Glycosil® and is in preclinical development as the HyStem®-Rx product line. Figure 3 illustrates the primary molecular structures present in HyStem®-C (also termed Carbylan-GSX or Extracel® in literature citations), in which thiol-modified gelatin is incorporated to provide cell adhesion sites required for delivery of viable cells in preclinical models and for future therapeutic use [18].

The inflammatory response of Carbylan-GSX (henceforth, HyStem®-C) has been characterized with specific cytokine expression levels *in vivo* and *in vitro*. HyStem®-C was been prophylactically injected unilaterally into bilaterally scarred rabbit vocal folds and saline injection was used as the sham treatment [11]. Rabbits were euthanized 1, 3, 5, 10 and 21 days post surgery and inflammatory cytokines were evaluated using real-time polymerase chain reaction (RT-PCR). There was an initial statistically significant upregulation of IL-6, IL-1 β and TNF- α on day 3 in HyStem®-C treated vocal folds over the saline treatment. This mild, short-term inflammatory response is not alarming, as all cytokine levels returned to baseline by day 21, and upregulation of TNF- α is expected as part of the native wound healing response. TNF- α imparts restorative effects during the early inflammatory response by recruiting macrophages and fibroblasts as well as by activating growth factor expression in multiple cell types (i.e., macrophages, keratinocytes and fibroblasts) [12]. When HyStem®-C was cultured with human vocal fold fibroblasts *in vitro*, evaluation of cytokines (i.e., cyclooxygenase 2: COX-2, IL-6, IL-8, TNF- α) mimicked the minimal inflammatory response found in the previous *in vivo* investigation. There was an upregulation of IL-8 and TNF- α only after day 7 of cell-hydrogel culture [9]. Further, immunocytochemical analysis of two markers for active inflammation in fibroblast culture (i.e., biosynthase prolyl 4-hydroxylase and intercellular adhesion molecule-1) showed no distributional differences between vocal fold fibroblasts cultured in the 3D HyStem®-C and Matrigel® conditions or the 2D HyStem®-C and polystyrene conditions.

An underlying mechanism for the favorable inflammatory profile of HyStem®-C has recently been elucidated. The immunophenotype of monocyte derived macrophages was evaluated following culture with three different cell types (i.e., bone marrow derived mesenchymal stromal cells: BM MSC, adipose derived mesenchymal stromal cells: AD MSC and vocal fold fibroblasts) and in two physical conditions (2D: tissue polystyrene and 3D: HyStem®-C) [10]. The least inflammatory profile overall was achieved when the monocyte-derived macrophages were cultured on the BM MSC-HyStem®-C and the AD MSC-HyStem®-C constructs. Flow cytometry revealed lower expression of CD16 and HLA-DR and higher expression of CD206 than the other conditions. These findings suggest that MSC encapsulated within HyStem®-C may be able to favorably modulate the inflammatory response *in vivo* through interaction with native monocytes and suggests its potential utility as a cell-based therapeutic for soft tissue regeneration.

3.2 Extracellular Matrix Remodeling Response

3.2.1. Physiology of matrix remodeling—During wound healing, post injury day 4–14 is marked by re-epithelialization, cell proliferation angiogenesis, and early matrix formation. During epithelialization, macrophages encourage fibroblast secretion of keratinocyte growth

factor and other molecules by stimulation with IL-1 and TNF- α [12]. Early matrix is generated through pathways involving fibroblasts, macrophages, PDGF, TNF- α and TGF- β 1. Fibroblasts at the injury site begin producing fibronectin, proteoglycans and glycosaminoglycans (GAGs) to create the provisional matrix which will eventually be replaced with collagen in the mature remodeling phase. This initial scaffold is compliant and allows for ease of cell migration (i.e., fibroblasts, neutrophils, macrophages) [12].

Approximately day 8 through at least 1 year following injury the ECM matures and remodels, which is potentially the most important phase clinically. TGF- β 1 is pivotal in directing matrix remodeling early in the phase, with peak levels occurring 7–14 days post injury for incisional wounds [12]. The provisional matrix scaffold is progressively replaced with collagen. The density of the collagen arrangement increases over time, which likely corresponds with increased matrix stiffness [24] and fluctuating mechanical load imparted to the fibroblasts. Once the collagen rich matrix is established, active cell migration slows. This can be partially attributed to the increased stiffness of the matrix, which imparts excessive mechanical tension on the fibroblasts [25]. Fibroblasts that are fully attached to the matrix undergo a phenotypic transformation into myofibroblasts and organize into focal adhesions; in this orientation they have the mechanical leverage to contract and close the wound with centripetal force.

An understanding of the ultrastructure of the native ECM of the vocal fold lamina propria is crucial for appreciating the deleterious potential of the mature wound healing response. The density of the native ECM architecture increases with lamina propria depth. Both the density and ECM composition dictates the mechanical properties of this tissue; from the gelatinous, loose and pliant SLP to the harder and strong DLP. Elastin is most dense in the intermediate lamina propria, and collagen is most dense in the DLP [26]. Elastin fibers, which make up approximately 9% of the tissue total protein in the human lamina propria, are oriented anterior to posterior along the membranous vocal fold [27]. In the vocal fold, elastin contributes mechanical resiliency to the native vocal fold; it allows the vocal fold to be stretched under a mechanical load and return to its original configuration. Collagen types 1, 2 and 3 are found in the lamina propria; their longitudinal orientation parallel to the vocal fold edge allows them to bear stress of the intrinsic laryngeal musculature as well as to provide strength [26, 28]. The interstitial proteins native to the lamina propria are oriented in a semi-layered arrangement. These proteoglycans (i.e., decorin, fibromodulin, aggrecan, versican) and GAGs (including HA, a non-sulfated GAG) and glycoproteins (i.e., fibronectin) are essential for controlling tissue viscosity, fluid content and collagen fiber density and size [29], which are all associated with the biomechanical properties of the tissue. Fibroblasts maintain the extracellular matrix of the lamina propria [30] which has motivated a recent clinical trial of the use of buccal fibroblast injections for attenuating vocal fold scarring [31].

Histological analysis of biopsy tissue from ten patients with vocal fold scarring revealed significant ECM variability between patients which was potentially a result of the depth of surgical insult and underlying etiology [32]. As scarred human vocal fold tissue is difficult to obtain for research purposes, animal models have been instrumental in providing researchers valuable information about the native ECM remodeling response following injury. It should be noted that the scarring procedure employed in these animal studies typically creates an injury that is deeper than those generally found in the clinical population. The traditional vocal fold stripping technique for animal models employs a small forceps which is used to clip the mid-membranous tissue through the epithelium, lamina propria and a part of the thyroarytenoid muscle [11, 33]. As vocal fold scarring in the clinical population does not typically involve the muscle and has a multitude of non-surgical origins, caution should be taken when extrapolating the findings from animal studies.

The lamina propria scarring response has been well characterized in rat, rabbit, canine and pig models since Hirano's seminal canine investigation [3, 8]. Briefly, an upregulation of procollagen 1 (a collagen precursor) is generally noted in the early stages of wound healing, which progresses to thickened, disorganized bundles of collagen by six months post injury [34–36]. At 2 and 6 months post injury, descriptions of elastin fibers include “shorter,” “thinner,” “less wavy,” “disorganized,” “fragmented” and “less dense” [34–36]. HA is crucially important for imparting space filling, shock absorption and tissue viscosity mediating effects [37] in the vocal folds. Its expression profile has been rigorously assessed in models of vocal fold scarring. HA levels are generally decreased as compared to unscarred controls from day 1 until approximately 15 days post injury [33–36, 38]. At 2 and 6 months post injury HA stabilizes to control levels but may have a different distribution throughout the lamina propria than in non-injured tissue [35, 36].

3.2.2. Role of hydrogels in matrix remodeling—Ideally, injectable hydrogels would both encourage self-regeneration of the tissue and degrade completely over time. As the mature phase of ECM remodeling can persist at least one year following injury, *in vivo* residency of a hydrogel until this time is reasonable; faster degradation times would also be appropriate if the hydrogel promotes significant and early self-repair of tissue. For example, Hylan-B has been reported to be present and providing ongoing therapeutic benefit in rabbit vocal fold tissue 12 months post injection [19]. The viscoelasticity of hydrogels will not likely be identically tuned to an individual's native vocal fold tissue properties. Therefore, a permanent hydrogel is not preferred because it could unfavorably interfere during vibration. Degradation time of injected hydrogels *in vivo* is affected by a number of factors including where the material is injected, whether cells are encapsulated within the gel and the degree of crosslinking of the material.

Collagen-HA composite gels evaluated as candidates for vocal fold use have demonstrated variable degradation times *in vitro*. These gels have been fabricated in various ways: unmodified HA has been entrapped in collagen gels physically [39], and chemically modified HA derivatives have been combined with mature (Type A) and immature (Type B) collagen fibrils [40]. In both studies, the collagen-HA gels were cultured with porcine vocal fold fibroblasts over a period of 28 days and had little evidence of new matrix production. While one type of collagen-HA gel had significant mass loss [39], the fibrillar collagen resisted gel compaction [40]. It is likely that the addition of the chemically modified HA reinforced the matrix and thereby prevented gel compaction. Indeed, crosslinked HA can prevent collagen contraction by a process termed “molecular stenting [41].”

Matrix remodeling has also been evaluated in polyethylene glycol diacrylate (PEGDA)-based hydrogels. These are biological “blank slates”, which allows them to be engineered to conform to tissue specific needs. Hahn and colleagues have investigated four PEGDA-based hydrogels of varied molecular weight, which have correspondingly altered mesh size and mechanical properties [42]. Porcine vocal fold fibroblasts were statically cultured within the hydrogels and collagen, and elastin and sulfated GAG content were later assessed. Interestingly, mesh size was a pivotal mediator in ECM synthesis; collagen and sulfated GAG levels were inversely related to initial mesh size. Sulfated GAG production was also affected by initial hydrogel modulus. The tunability of biomaterials is an exciting prospect for vocal fold bioengineers.

HA-based hydrogels have demonstrated promise *in vitro* and *in vivo* for favorable matrix remodeling. In the absence of injury, Hylaform® (Hylan B) injection into rabbit lamina propria yielded newly formed connective tissue (i.e., collagen, HA) and fibroblast migration within 1 month [19].

HyStem®-C (Carbylan-GSX) has also demonstrated a capacity for ECM remodeling. Human vocal fold fibroblasts cultured in HyStem®-C had reduced collagen type 1 expression and increased fibronectin expression over cells cultured in Matrigel (3D) [9]. HyStem®-C is thought to repair vocal fold scar *in vivo* by mediating collagen deposition, cell attachment and migration of fibroblasts [43]. It has been documented that the restorative effects of mesenchymal stromal cells (MSC) are amplified when they are delivered to scarred vocal fold tissue within an appropriate scaffold. The thiol-modified gelatin in HyStem®-C provides sites for cell adhesion. *In vivo* comparison of treatment of established rabbit vocal scar with bone marrow-derived MSC+ HyStem®-C vs. cells alone vs. hydrogel alone indicated that the combination therapy yielded enhanced expression levels of fibronectin, procollagen 3 and TGF β -1. TGF- β 1 is a critical player for early matrix production: (i) it activates macrophages and fibroblasts which encourages fibroblast proliferation and additional matrix production; (ii) it recruits tissue inhibitor of metalloproteinases (TIMP) which prevents collagen degradation; and (iii) and it encourages fibronectin secretion which facilitates cell-matrix adhesion [12].

3.3 Biomechanical Response

3.3.1 Connecting physiology with biomechanics—Within the vocal fold lamina propria, the ECM architecture is translated into the most important clinical manifestation of scarring- tissue viscoelasticity. Phonation threshold pressure (PTP), the minimum amount of subglottic air pressure needed to initiate vocal fold oscillation, is a commonly used clinical tool to provide an objective indication of the subjective sensation of vocal effort. PTP is directly related to the viscosity of the vocal fold mucosa according to theoretical predictions and studies using excised canine larynges, a physical model of vocal fold mucosa, as well as in human subjects [44–46]. Further, altered viscoelastic properties of the vocal fold lamina propria could cause impaired mucosal wave or the vocal folds to vibrate out of phase with one another, both of which could negatively influence vocal quality.

The fibrous proteins (i.e., collagen, elastin) contribute tensile stress/strain properties to the tissue, and the interstitial proteins (i.e., proteoglycans, GAGs) contribute viscoelastic properties. During vibration, there is an upward surface propagation of a vertical wave of shear stress in the vocal fold mucosa. The application of the parallel plate rheometer to impart sinusoidal oscillatory shear stress to biomaterials and excised vocal fold tissue has made inroads into capturing functional outcomes of bioengineered therapeutics [47–53]. Viscoelastic properties of human vocal fold mucosa using a parallel plate rheometer have previously been reported [54]. In this study, biomechanical properties varied with gender and age, which corresponded with ECM data [26, 55]. One limitation of traditional parallel plate rheology for assessing vocal fold tissue and injectables is that the frequency range over which the measurements are valid has generally been limited by the inertial effects of the material. As such, measurements have typically been reported for a sub-physiologic frequency range. Introduction of an alternative method for appreciating viscoelastic shear properties at physiological frequencies has recently been suggested [56].

Briefly, the functions of the proteoglycans and GAGs found in the vocal lamina propria are crucial for imparting tissue viscosity, space filling and shock absorption in this biomechanically rich environment [29]. Decorin (in SLP) and fibromodulin (in DLP) are small proteoglycans which bind to collagen and encourage delayed or thinner fibril development. Aggrecan and versican are larger proteins which are able to bind molecules, and thereby contribute capacities for shock absorption and space filling. HA has an impressive ability to bind water molecules and fill space. The precise mechanisms by which the ECM arrangement is translated into their viscoelastic properties is not yet fully

understood. Successful vocal fold lamina propria intervention requires a biomaterial which is biomechanically similar to native vocal fold mucosa.

3.3.2 The role of the hydrogel in biomechanics—Many of the injectable gels intended for vocal fold lamina propria injection include HA as a critical component. Endogenous HA is known to contribute favorably to native vocal fold viscoelastic properties; excised human vocal fold tissue that had undergone HA digestion had a 35% reduction in elastic shear modulus and 70% increase in dynamic viscosity as compared with normal tissue [57]. Investigation of the potential use of HA and its derivatives as bioimplants suggested that at concentrations of 0.5–1% (w/v), injectable HA had similar viscous shear properties as adult human vocal fold mucosa [49].

Prior to injection, Hylaform© and HyStem-C® possess similar viscoelastic properties to cadaveric vocal fold mucosa as determined by parallel plate rheology [47](see Figure 4; HyStem-C® is referred to here as “Carbylan GSX 5%”). This mechano-mimicry corresponded to similar findings during interaction with native tissue. Hylaform© which was injected into uninjured rabbit vocal fold yielded improved viscoelastic properties over vocal folds injected with Teflon and crosslinked collagen (Zyplast) in the short-term [52] and long-term [20]. When HyStem-C® was applied prophylactically in a rabbit scarring model, improved biomechanical properties (i.e., reduced elastic modulus and viscous modulus) of the vocal fold tissue were observed as compared to untreated controls after 3 weeks [58] and 6 months [59].

Jia and colleagues have investigated the biomechanical properties of photo-crosslinkable HA-based hydrogels [60]. Sodium periodate with glycidyl methacrylate (HAox/GMA) had the lowest elastic stiffness and viscosity measurements of the investigated hydrogel formulations, as determined by an acoustic shaker technique at physiological frequencies (20–180 Hz). Jia and collaborators have also developed HA-based soft microgels and crosslinked microgel networks intended for vocal fold lamina propria which have demonstrated an impressive capacity for biomechanical tunability to native vocal fold properties through physiochemical engineering [61].

PEG30, created by photopolymerization of PEG diacrylate in the presence of PEG, was injected unilaterally and superficially into canine vocal folds [62]. Average phonation threshold pressure (PTP) values were found to be low (4–8.5 cm H₂O) and did not vary statistically from zero to twelve weeks post injection. These PTP measurements were obtained through an *in vivo*, manually elicited paradigm that allows for testing at multiple time points in the same animal.

Drug delivery has also been explored by injecting a hepatocyte growth factor (HGF) containing hydrogel into the vocal folds in an acute scarring model in canine [63] and a chronic scarring model in rabbit [64]. Histological analysis revealed that in the HGF-treated acute scar group, HA and elastin levels were restored in the superficial lamina propria and minimal collagen had accumulated [63]. Levels of elastin and HA of treated, mature scar were similar to unscarred vocal folds, while disorganized collagen was found [64]. Minimal tissue contraction was observed in both treated acute and mature scar [63, 64].

Hydrogels have also been designed to treat deeper tissue defects. Using a combination tailored to the unique biomechanical properties of the vocal folds, methacrylated HA-based gels for the mucosa and PEG diacrylate-based gels for the vocal fold ligament were investigated [65]. These living hydrogels were designed to be photopolymerized *in situ*. Tensile testing of the materials intended for the ligament revealed stress-strain responses of the gels which were similar to the native ligament at low strains only; rheological testing of

the materials intended for the mucosa demonstrated reasonable dynamic viscosity across the frequency range, but elastic shear moduli that were several fold higher than is found in native tissue.

A clinical trial of an auto-crosslinked hyaluronan gel demonstrated that improved tissue biomechanics (as judged by videostroboscopy) translated to improved perceptual ratings of voice [66]. Thirty-eight patients received injection of the gel into either the lamina propria or the thyroarytenoid muscle, depending on their need for treatment of vocal fold scarring/sulcus, or to close the glottal gap. At three and twelve months post-operatively, patients demonstrated significant improvement in glottal closure, mucosal wave as well as for perceptual judgments of their voices. Importantly, the Voice Handicap Index-10 (VHI-10) was also used as a self-assessment to track the impact of the voice disorder on one's life. Significantly improved scores on the VHI-10 were reported for 94.3% of patients at three months post-operatively, and 79.2% of patients twelve months post-operatively.

4. Conclusion

The array of biomimetic materials which are currently being investigated in the laboratory have the power to usher in a new era of treatment options for vocal fold scarring in the clinic. Biocompatible hydrogels, in particular those based on HA and designed for clinical use, have the ability to modulate and enhance the wound healing response in injured vocal folds. Moreover, for use in cell therapy of vocal folds, injectable living hydrogels permit delivery and retention of therapeutic cell populations into the tissue. Large scale, multi-center randomized controlled trials are being planned to assess the clinical utility of HyStem®-C as both a stand-alone device and a combination cell-material product. As vocal fold scarring is not a life-threatening diagnosis, demonstration of safety and biocompatibility in Phase I trials will be of particular importance. Cell biodistribution studies will also be required to document whether cell migration occurs distal to the local injection site. Finally, clinical grade material generated according to current Good Manufacturing Practices (cGMP) is required prior to testing in human populations; this is underway. The collaboration between voice scientists, voice care practitioners, chemists, bioengineers, and the biotechnology industry is critical for creating safe and clinically valid solutions for patients with vocal fold scarring.

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Figure 1. Normal rat vocal fold

Coronal histological image of the mid-membranous rat vocal fold stained with hematoxylin and eosin. Superficial to deep, the layers of the vocal fold include the stratified squamous epithelium (**E**), the lamina propria (**LP**) and thyroarytenoid muscle (**M**).

Figure 2. Scarred rat vocal fold

Coronal histological image of the midmembranous rat vocal fold three days following a scarification injury (hematoxylin and eosin stain). The specimen demonstrates the early inflammatory response associated with injury. There is some evidence of granulation, but significant fibrosis has not yet occurred. The epithelium is thick and irregular and the basal cells are disorganized. The lamina propria is more cellular than found in Figure 1, and contains increased fibroblasts (**F**) and inflammatory cells including neutrophils (**N**) and macrophages (**M**). The extracellular matrix (**ECM**) also appears to be altered as compared to Figure 1. A few small, thin walled vessels are present which represents angiogenesis (**A**).

Figure 3. Chemistry of HyStem[®]-C formation

The crosslinking of thiol-modified gelatin (Gelin-S[®]) and CMHA-S (Glycosil[®]) occurs within 10–30 minutes at pH 7.4 and does not alter the viability of cells nor affect peripheral tissues.

Figure 4. Rheological characterization of various vocal fold injectables

Reprinted with permission from *The Laryngoscope*, 117(3):516–521 ([47]). Viscoelasticity of hyaluronan and nonhyaluronan based vocal fold injectables: implications for mucosal versus muscle use. Shear stress applied by a parallel plate rheometer evaluated the viscoelastic properties of four vocal fold injectables prior to injection. Cadaveric vocal fold mucosa data is present for comparison. Data includes mean elastic shear moduli (A), mean viscous shear moduli (B), and mean dynamic viscosities (C).

Table 1

Experimental and Clinical Hydrogels for the Vocal Fold Lamina Propria

BIOMATERIAL	REPORTED INDICATION	INFLAMMATORY RESPONSE	ECM RESPONSE	BIOMECHANICS
Hylaform® (Hylan-B Gel)	Lamina propria scarring/ muscle bulking	None (human, rabbit)	12 mo= HA, ↑ collagen production (Ref. 19)	Rheology; Similar ES, VS, DV to human vf mucosa (Ref. 47)
HyStem®-C (Carbylan-GSX)	Lamina propria scarring	Mild response, resolved by day 21 (rabbit) (Ref. 11)	Day 5= ↑ procollagen 1, Fn, TGF- β1 exp (Ref. 43); 6 mo= ↓ Fn, Fn, TGF-β1, procollagen 1 (Ref. 59)	Rheology; Similar ES, VS, DV to human vf mucosa (Ref. 47)
Restylane®	Lamina propria scarring/ muscle bulking	Mild response at 1 week and ↓ at 3 mo. (rabbit) (Ref. 22)		Rheology; ES, VS and DV values are mildly ↑ over Hylan- B and Carbylan- GSX (HyStem- C)(Ref. 47)
Autocrosslinked HA gel (Ref. 66)	Lamina propria scarring/ muscle bulking	No inflammatory response was reported in 38 patients treated	-	Improved observations of tissue biomechanics noted on videostroboscopy 3 and 12 mo. post operatively.
Photo- crosslinkable methacrylated HA hydrogels (Ref. 60)	Lamina propria scarring	-	-	Acoustic shaker method; indicated HAox/GMA had lowest ES and DV of the formulations tested.
HA-based microgels (Refs. 56, 61)	Lamina propria scarring	HAADH/HAALD microgels are non- toxic	-	Tuneable viscoelastic properties
Collagen- alginate hydrogel (Ref. 39)	Lamina propria scarring	-	New collagen reticular fibers and sulfated GAG by day 28 in culture with porcine VFF	-
Collagen-HA hydrogels (Type A & B) (Ref. 40)	Lamina propria scarring and more severe vocal fold diseases	-	Minimal HA and collagen production over 28 day culture with VFF	Torsional wave analysis: Type A with VFF-Initial ↓ then moderate ↑ in elastic modulus over time. Type B with VFF- ↑ elastic modulus over time.
Two layer hydrogel system (methacrylated HA/ diacrylated PEG) (Ref.65)	2 layer hydrogel (targeting the mucosa and the vocal ligament)	-	Collagen/sulfated GAGs produced at 14, 28 days in culture with human dermal fibroblasts	Mucosa hydrogel: DV similar to human vf mucosa; ES ↑ than human vf mucosa; ligament hydrogel: stress- strain response similar to ligament at low strain only.
PEGDA-based hydrogels (Ref. 42)	Lamina propria scarring	-	Collagen and sulfated GAG ↑ with ↓ mesh size in culture with porcine VFF	DMA test; Elastic compressive modulus ↑ as PEGDA mol. wt. ↓
PEG30 (Ref. 62)	Lamina propria scarring	No clinical evidence of inflammation (erythema, edema) noted; significant macrophage infiltration 1 mo. post injection (canine)	In uninjured tissue, slight to mild fibrosis observed in 1/3 canines 1 mo. post injection	Rheology; ES at 10 Hz is within or ↓ human vf mucosa. PTP did not differ between preinjection and final recording sessions during manually elicited phonation.
Hepatocyte Growth Factor- Drug Delivery System (HGF- DDS)	Lamina propria scarring	-	Elastin and HA of treated, mature scar similar to unscarred vf; Disorganized collagen in treated, mature scar (Ref.	PTP significantly ↓ in treated versus sham group in mature scar (Ref. 64).

BIOMATERIAL	REPORTED INDICATION	INFLAMMATORY RESPONSE	ECM RESPONSE	BIOMECHANICS
			64). Minimal tissue contraction noted in treated acute (Ref. 63) and mature scar (Ref. 64).	

Legend: elastic shear moduli (ES), viscous shear moduli (VS), dynamic viscosity (DV), expression (exp), fibronectin (Fn), fibromodulin (Fm), vocal fold (vf), vocal fold fibroblasts (VFF), phonation threshold pressure (PTP)