THE IMPACT OF MECHANICAL PROPERTIES OF POLY (ETHYLENE GLYCOL) HYDROGELS ON VOCAL FOLD FIBROBLASTS' BEHAVIOR

A Thesis

by

HUIMIN LIAO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2007

Major Subject: Chemical Engineering

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Approved by:

Chair of Committee,	Mariah Hahn
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Head of Department,	N. K. Anand

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ABSTRACT

The Impact of Mechanical Properties of Poly(ethylene Glycol)
Hydrogels on Vocal Fold Fibroblasts' Behavior. (May 2007)
Huimin Liao, B.S., Fudan University;
M.S., National University of Singapore
Chair of Advisory Committee: Dr. Mariah Hahn

Vocal fold scarring, caused by injury and inflammation, presents significant treatment challenges. Tissue engineering might be a promising treatment for vocal fold restoration or regeneration. It is important to investigate how scaffold properties alter cell behavior instead of screening thousand of materials, which is fundamental knowledge for rational scaffold design. This work studies how tuning only one parameter, mechanical strength of the hydrogel scaffold, influences the extracellular matrix production of encapsulated porcine vocal fold fibroblast (PVFF). PVFF cells were encapsulated by photopolymerization in 10 wt%, 20 wt%, and 30 wt% poly(ethylene glycol) diacrylate (PEGDA) hydrogels (MW 10,000), with the similar biochemical environment and network structure but different mechanical properties. Cell adhesive peptide, RGDS, was grafted into each hydrogel network to mimic a cell adhesive environment. The glycosaminoglycans (GAGs) production per cell increased from 10 wt% to 20 wt%, 30 wt% gels, with an increase in hydrogel stiffness. The collagen production per cell increased from 10 wt% to 20 wt% gels but no further increase occurred with the increasing modulus from 20 wt% to 30 wt% gels. Interestingly, in hydrogels of intermediate modulus (20% PEGDA hydrogels), the highest elastin per cell was observed compared with gels with higher and lower storage modulus after day 30. Histological analysis showed GAGs, collagen and

elastin were distributed pericellularly. However, the organization of collagen type I appeared to be influenced by gel mechanical properties, which was confirmed by immunohistological analysis. Furthermore, the immunohistological analysis showed that the phenotype of PVFF is regulated by the stiffness of the PEG hydrogel. This study demonstrates that different levels of VFF ECM formation may be achieved by varying the mechanical properties of PEG hydrogels and validates a systematic and controlled platform for further research of cell-biomaterials interaction.

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On a personal note, I am grateful to my friends and family who are supporting me in the pursuit of my goals. Lastly, to Zhiwen Zhang, thank you for your neverending support and patience in times of frustration and making me smile.

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1. INTRODUCTION FOR VOCAL FOLD SCARRING AND RESTORATION

1.1. The Anatomy of Vocal Fold

Voice quality is dependent on the unique layered ultrastructure of the vocal fold [1]. Histologically, the human vocal fold is composed of three distinct layers (Figure 1): 1) the "cover", which includes the outer epithelia and superficial lamina propria (SLP); 2) the vocal "ligament", including intermediate lamina propria (ILP) and deep lamina propria (DLP); and 3) the body, composed of thyroarytenoid muscle [1, 2]. The SLP is composed of interstitial proteins, such as fibronectin, and a relatively low density of collagen and elastin fibers. On the other hand, the intermediate lamina propria and deep lamina propria are composed of well-oriented of collagen and elastin fibers, with a relatively low density of interstitial proteins [3, 4]. The lamina propria has been described as viscoelastic [2, 4, 5], and the elastic and viscous shear properties of lamina propria must be in appropriate balance for normal vocal fold vibration [2].

Generally, scarring changes the viscoelastic structure of the lamina propria, causing an increase in stiffness so that the normal mucosal wave is disrupted during phonation. This results in hoarseness or raspy voice, increased vocal effort, voice breaks, vocal fatigue, and possibly breathy voice quality [2 - 5]. Study has shown that vocal fold scarring is most frequently limited to the SLP [2]. Developing a tissue-engineered construct that mimics SLP behavior is therefore important to the vocal fold scar restoration.

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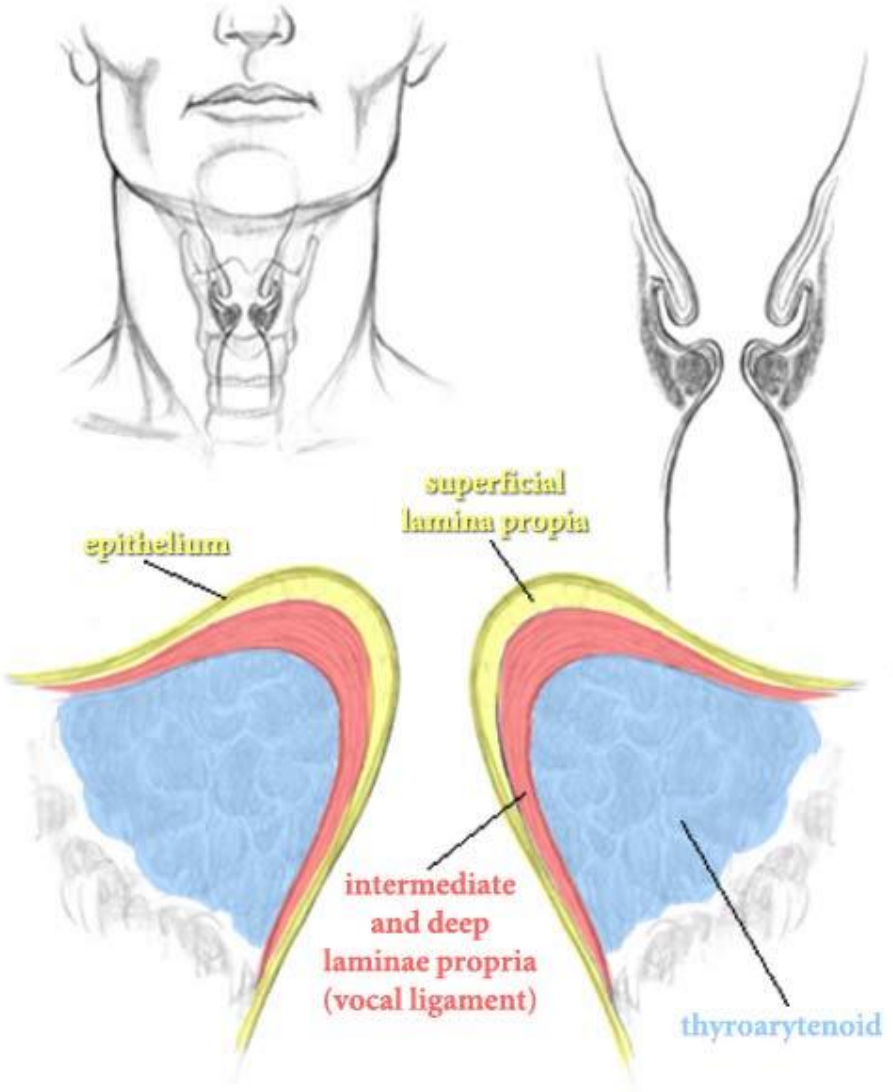
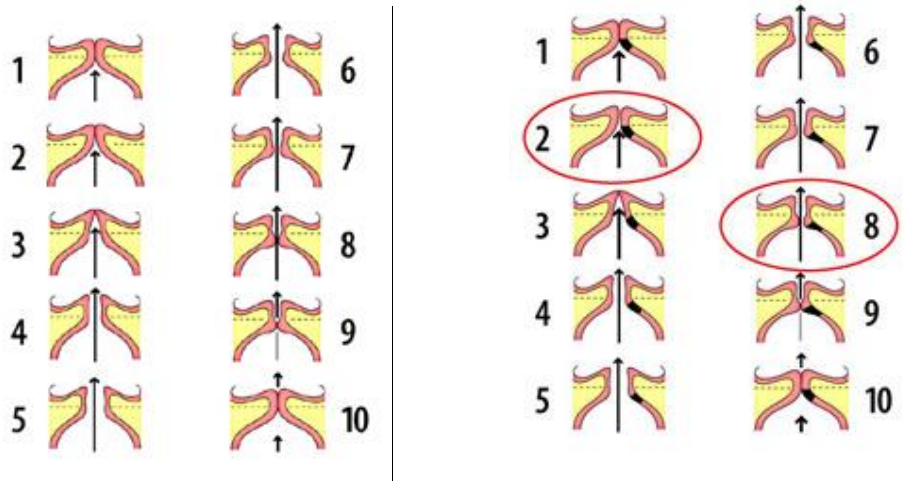


Figure 1. The structure of human vocal fold. [1]

1.2. Vocal Fold Scarring

In the world, it is estimated that from 3% to 9% of population suffer from vocal fold disorders across the lifespan [6]. Vocal fold scarring, whose formation is induced in response to injury or inflammation, is the greatest cause of poor voice and presents significant treatment challenges [6 - 9]. Figures 2 (a) and (b) compare the vibratory cycles in normal and scarred vocal folds respectively. In voice production, air is

forced out from the lungs. This fast-moving air stream produces a Bernoulli Effect as it encounters the vocal fold: the induced low pressure causes the bottom of the vocal fold to close, followed by the top. This closure of the vocal fold cuts off the air flow and releases a pulse of air, completing a vibratory cycle. Scarring hampers the vibratory cycle, resulting in incomplete closure of the vocal folds (known as glottal insufficiency) and hence poor voice (panel 2 and panel 8 in Figure 2 (b)) [1-2, 7].



(a) (b)

Figure 2. (a)Vibration of normal vocal fold tissue. (b) Vibration of scarring vocal fold tissue. ◆ represents scarring tissue. [1]

Research has shown that scarring increases the stiffness of the lamina propria, in particular that of the SLP, so that the normal mucosal wave is disrupted during phonation, which results in hoarseness or raspy voice, increased vocal effort, voice breaks, vocal fatigue, and possibly breathy voice quality [6 - 9].

1.3. Extracellular Matrix Composition of Normal and Scarred Lamina Propria

The extracellular matrix (ECM) does not only provide structure and support for tissue,

but also promotes cell adhesion, migration, growth, differentiation and activates intracellular signaling [2, 10]. The ECM of the lamina propria includes fibrillar proteins, such as collagen and elastin, interstitial proteins such as fibronectin, and proteoglycans and glycosaminoglycans (Figure 3). Fibroblasts, the primary cell type composing the lamina propria, contribute to the deposition, degradation and rearrangement of ECM [11]. Collagen is one of the most important tissue structural proteins and is critical to bearing stress and resisting deformation during phonation [2, 12]. Collagen Type I is the most ubiquitous fibrillar collagen in both the skin and the vocal folds, providing a structure of high tensile strength [13]. Elastin, which is sparse in the SLP [7, 9], is made of highly hydrophobic crosslinked proteins and enables the tissue to return to its original shape after deformation [2]. Fibronectin, an ECM glycoprotein, is an important adhesion molecular, which contributes to matrix organization and wound healing. Another important ECM component is glycosaminoglycans (GAGs), unbranched polysaccharide chains composed of repeating disaccharide units. Since GAGs are negatively charged and strongly hydrophilic, they allow the tissue to absorb large amount of water and to withstand large compressive forces [2].

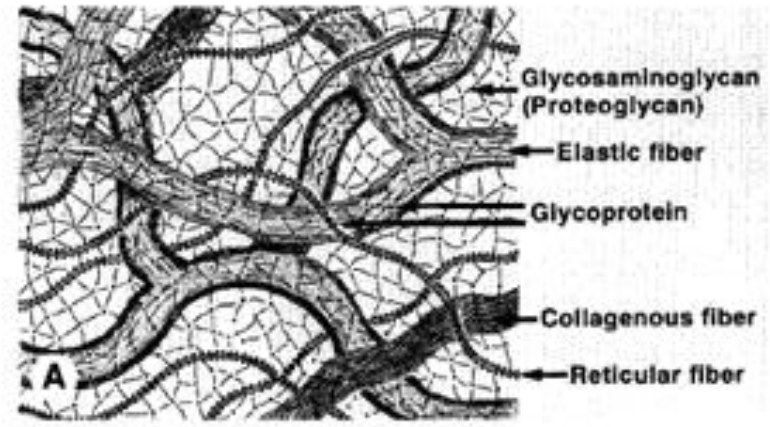


Figure 3. Schematic of ECM in SLP of lamina propria of younger adult. [14]

Studies in animal models have shown that the density and organization of the ECM components determine the biomechanical properties and vibratory behavior of the lamina propria and that the ECM distribution and density are changed in both acute and chronic vocal fold scarring (Table 1) [6 - 9]. In scarred vocal folds, collagen lacks its characteristic organization into parallel bundles [8, 10, 15]. In addition, it appears that elastin is frequently less dense and more disorganized in scarred vocal folds [16]. A decrease in GAG and water in the ECM in scarred vocal folds has been observed [2]. The development of treatment for vocal fold scarring must be based on the comprehensive understanding regarding the roles of ECM proteins in vocal fold vibratory function.

Table 1. Summary of Histologic Changes in ECM Components in Scarred Vocal Folds of Different Animal Models. [8]

	Injured Rabbit Vocal Fold						Injured Canine Vocal Fold		Injured Pig Vocal Fold			Injured Rat Vocal Fold			
ECM component	3d	5d	10d	15d	2 mo	6 mo	2 mo	6 mo	3d	10d	15d	2wk	1 mo	2 mo	3 mo
Procollagen					↑	↔	↑	↔							
Collagen					↓	↑	↔	↑	↔	↔	↑	↑	↑	↑	↑
Elastin					↓	↔	↓	↓							
Fibronectin					↑		↑	↑				↑	↑	↔	↔
Hyaluronan	↓	↔	↓	↓	↔	↔	↔	↔	↓	↓	↓	↓	↓	↓	↓

↑ = increase; ↓ =decrease; ↔ = no change; blank = not measured

1.4. Phenotype of Fibroblasts in Lamina Propria

Fibroblasts are the primary cell type in normal lamina propria, expressing vimentin [2, 13, 17, 18]. Fibroblasts in scarred lamina propria change their phenotype into myofibroblasts, which have characteristic of both smooth muscle cells and fibroblasts [13]. Myofibroblasts show increased production of matrix proteins, functioning to repair injuries in the lamina propria [2]. Studies have demonstrated environmental cues tend to stimulate fibroblast differentiation into proto-myofibroblasts, an intermediary cell type between fibroblasts and true myofibroblasts, expressing smooth muscle α-actin (SMα-actin) and vimentin at the same time [18]. The proto-myfibroblasts differentiate into myofibroblasts by the stimuli of growth factors, newly synthesized ECM proteins and mechanical loading.

1.5. Current Treatment for Vocal Fold Scarring

The goals of treatments for lamina propria scarring are to: 1) restore normal lamina propria pliability and geometry; 2) reduce the force associated with voice production and vocal fatigue; 3) improve overall voice quality [1]. To soften or replace scarred tissue, some medical treatments are being developed for vocal fold scarring. However, current clinical treatments produce inconsistent and suboptimal results, which makes

vocal fold scarring a major problem awaiting improvement in the future [8].

Speech therapy is an important preliminary step in treatment of patients with vocal fold scarring [8, 9]. Speech therapy trains patients to use appropriate voice behavior and assists the patients in compensating for the voice problems. A limitation of speech therapy is that it does not directly change the histological nature of the scarred vocal folds so that it cannot restore function to scarred tissue, only improving the manner in which it is used.

Surgical treatments are performed to improve glottal insufficiency during voice production [8, 9]. This approach enables the better vocal fold closure and better vocal fold vibration. However, the scarred tissue remains in the lamina propria and the normal vibratory properties of the vocal folds cannot be restored.

Biomaterial injection into the SLP is the most popular clinical treatment currently. Bovine collagen, autologous collagen, autologous fat and hyaluronan (HA) have been injected into scarring tissue [8, 9, 19 - 24], with the aim of augmenting the lamina propria and softening the scarred tissue (Table 2). However, the success of such implants to achieve normal viscoelastic properties of vocal folds has been limited. In addition, resorption of injectable materials over time is another issue which results in implant failure.

Table 2. Summary of Studies on Biomaterial Injection [7]

Type of treatment	**Reference**	**Outcome**
Homologous collagen matrix injected in scarred vocal folds in rabbits	Kriesel K *et al.*	*Homologous collagen matrix simulated production of high quality procollagen *Homologous collagen matrix did not improve the biomechanical properties of scarred vocal fold
Collagen treatment of scarred vocal fold: case studies	Bjorck G *et al.*	*All patient had improved voice vibration after treatment *3 out of 4 patients had increased amplitude of vibration *2 out of 4 patients' voice quality had been improved *None of patients had experienced any side effect.
Autologous fat implantation for vocal fold scar	Neuenschwander MC *et al.*	* Patients had shown improvement on vocal fold function and quality of voice after injection with autologous fat
Autologous fat implantation into the vibratory margin for vocal fold scar	Hsiung MW *et al.*	* 7 out of 13 patients had improved in vocal fold function, 2 of them showed failure after 2 and 3 months, 2 were lost to follow-up and 2 showed no change.
Hyaluronic acid(HA) injection in rabbit model: Short-time study	Hertegard S *et al.*	* HA might alter viscoelasticity scarred vocal folds after 8 weeks *There was large variation in stiffness between two types of hyaluronan
Cross-linked hyaluronan for treatment of glottal insufficiency	Hertegard S *et al.*	* The patients showed better vocal fold status and vocal function after 12 months treatment. * Less resorption of hylan B gel was observed at the injected vocal fold edge * 3 patients showed temporary inflammation, which resolved without sequelae.

Growth factor therapy might be a potential treatment for vocal fold scarring by modifying fibroblast activity and reconstructing ECM of the vocal fold to improve vibration. Hirano S et al. applied hepatocyte growth factor (HGF) to the scarred vocal folds in a canine model since HGF has strong anti-fibrotic activity [25]. After

6 month treatment with HGF, less deposition of collagen, a decrease in elastin, less contraction of lamina propria and better mucosal wave amplitude were observed in treated vocal folds compared with untreated scarred vocal folds. However, vocal function was still reduced in the treated group as compared with the normal group.

2. TISSUE ENGINEERING

2.1. Tissue Engineering Approach

Tissue engineering has been demonstrated to be a powerful general strategy for tissue regeneration [26, 27, 28] and has great potential to restore function to scarred lamina propria [7]. Three important elements are manipulated in tissue engineering: (1) scaffolds, (2) cells and (3) regulatory factors (Figure 4). The most common strategy in tissue engineering is to use a scaffold to support, guide and simulate living cells to regenerate tissues. Changes in scaffold features, like network structure, mechanical properties or biochemical properties, can cause different cell responses [27, 29]. Vocal fold scarring might be eventually cured by using scaffolds, cells and regulatory factors together appropriately [9].

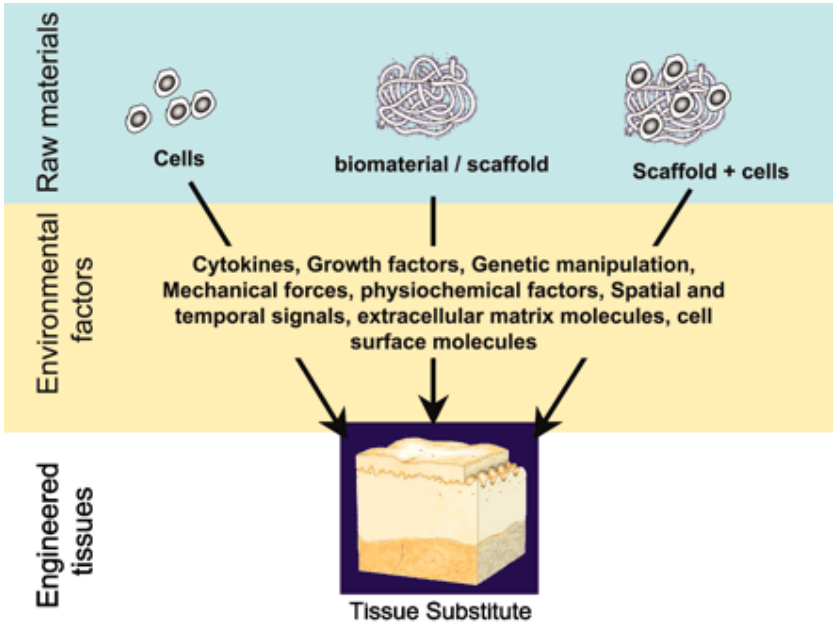


Figure 4. Tissue engineering approaches. Tissue engineering approaches are classified into three categories: (i) cells alone, (ii) cells with scaffolds, and (iii) scaffolds alone. Each one of these approaches can be enhanced by in vitro microenvironmental factors before application as a tissue substitute. [24]

Researchers have attempted to engineer tissue substitutes for skin, cartilage, bone, blood vessels, nerve, cornea, liver and kidney [26, 27]. Skin may be the most successful tissue engineered substance, and there are several FDA approved products in the market (i.e. *TransCyte* from Advanced Tissue Sciences, *Apligraf* from Organogenesis) [28]. In addition, tissue engineering of bone and cartilage has also experienced relative success [26 - 28]. Tissue engineering approach might have great potential to restore vocal function and generate the vocal folds.

Hahn MS *et al.* investigated collagen-alginate hydrogels seeded with fibroblast *in vitro* as a potential biomaterial for vocal fold restoration [30]. Collagen-alginate hydrogels resisted scaffold compaction and maintained their original shape and mass through 42 days in culture while allowing cells to synthesize new ECM. Kanemaru S et al. reported the use of mesenchymal stem cells (MSC) to treat vocal fold scarring [31]. MSCs were incubated in 1% hydrochloric acid atelocollagen then injected into injured vocal folds in a canine model. It was found 3-dimensional incubated MSCs promoted wound healing in terms of vocal fold appearance and histology. However, it is unclear how implanted cells acted in the vocal folds and controlled methods to induce appropriate stem cell differentiation are critical to obtain ideal results.

2.2. Challenges in Current Scaffold Engineering

Despite significant success in skin and cartilage tissue engineering, there are a number of challenges remaining in “off-the-shelf” tissue production. For example, available tissue engineered skin products only act as passive wound covers, lacking essential function and components of native skin [28]. Today, although researchers have gained more and more new knowledge, there are deficits in fundamental

understanding in tissue engineering. For instance, those related to structural and mechanical aspects of tissues, the behavior of biomaterials, and the cell-material interactions [26 - 28]. Furthermore, there is little progress toward a systematization of tissue engineering through the development of a foundation of broadly applicable theoretical principles relating cell behavior to scaffold material properties. However, such a foundation is needed for rational scaffold design [29].

As a consequence, there is a great demand to understand the complex interactions between scaffold features and cell responses so that cell response can be optimized by rationally tuning the scaffold material. However, scaffold features are usually interdependent, and this makes it difficult to investigate the dependence of cell response on isolated scaffold properties. For example, the mesh size of hydrogel network is interdependent with mechanical properties so that the cell response to the hydrogel results from both parameters [32 - 34]. It is necessary to create a 3-D platform in which the impact of isolated material properties on cell behavior can be systematically explored.

2.3. Proposed 3D Platform

Poly(ethylene glycol) (PEG) hydrogels have been chosen to fabricate the platform. The advantages of PEG-based hydrogels in tissue engineering are many: 1) PEG hydrogels are biocompatible. Thus the cells could be encapsulated in hydrogels without compromising their viability [35 - 38]; 2) The semi-permeability of PEG hydrogels allows nutrients, wastes, dissolved gases and water-soluble metabolites to pass through the gels while inhibiting the infiltration of the components of the immune system, thereby preventing the rejection of transplanted cells by the host's

immune system [39, 40]; 3) The water content and mechanical properties of PEG hydrogels can be tailored to be similar with some soft tissue (i.e. cartilage) by adjusting the concentration and molecular weight of PEG macromers [33, 35, 41]; 4) PEG hydrogels are essentially non-biodegradable. However, it is easy to modify PEG macromer backbone to create PEG derivatives with predictable and controllable degradation times [33, 35, 37, 42]; 5) Due to hydrophility and steric hindrances caused by mobile PEG chains, PEG hydrogels are intrinsically resistant to protein adsorption and cell adhesion, creating a biological blank "slate" into which desired bioactivity can be incorporated [43, 44]. Thus, the biochemical stimuli experienced by the cell can be tightly controlled. And 6) the mesh size and mechanical properties of PEG hydrogels can be tuned in an uncoupled manner.

This work will use PEGDA hydrogels to study the effect of isolated mechanical properties on vocal fold fibroblast's cellular ECM production. Since fibronectin is an important lamina propria interstitial protein and has significant functions in wound healing and cell adhesion, cell adhesion peptide Arg-Gly-Asp (RGD) [2], will be immobilized into poly(ethylene glycol) hydrogels in the present work to create materials that mimic some biochemical properties of the native ECM [38]. Although significant research has been conducted on the effects of the hydrogel properties on cell response [32, 33, 34, 38], the effects of isolated material properties (e.g., mechanical properties alterations independent of biochemical or microstructural changes) on cell behavior have not been explored. The broad thematic purpose of this research is to create a systematic and controlled approach to identify optimal scaffold parameters for scarred vocal fold restoration and regeneration. Our work should make significant contributions to help tissue engineering become a viable method for vocal fold scarring

treatment.

3. MATERIALS AND METHODS

The purpose of the current study was to elucidate the relationship between biomechanical properties of PEG hydrogel scaffold and ECM synthesis of porcine vocal fold fibroblasts (PVFF) when encapsulated in PEG hydrogels with immobilized RGDS peptides. PVFF were photoencapsulated in PEGDA (MW 10,000 Da) hydrogels of similar mesh sizes but varying biomechanical properties. The biochemical composition and distribution of ECM in cell-hydrogel constructs was examined as a function of the gel mechanical properties.

3.1. Synthesis of PEGDA

PEG diacrylate was synthesized by mixing 0.1 mmol/ml dry PEG (MW 10,000 Da, Fluka, Milwaukee, WI), 0.4 mmol/ml acryloyl chloride (ACRŌS, Geel, Belgium) and 0.2 mmol/ml triethylamine (J.T. Baker, Phillipsburg, NJ) in anhydrous dichloromethane (DCM) and stirring in argon environment overnight. The resultant solution was washed with 2 M K_2CO_3 solution and separated into two phases: aqueous and organic DCM phases. The DCM phase was collected and dried by anhydrous $MgSO_4$ (Fisher Scientific, Fair Lawn, NJ). Subsequently PEGDA was precipitated in diethyl ether (Fisher Scientific, Fair Lawn, NJ), filtered, and dried under vacuum at room temperature.

3.2. Acrylation of RGDS

The peptide RGDS (American Peptide, Sunnyvale, CA) was reacted with acryloyl-PEG-N-hydroxysuccinimide (ACRL-PEG-NHS, MW 3400 Da; Nektar, Huntsville, AL) in 50 mM sodium bicarbonate buffer (pH = 8.5) at 1:1 molar ratio for at least 2 h. The synthesized ACRL-PEG-RGDS was purified by dialysis, lyophilized

and stored at -80˚C until use.

3.3. Mesh Size Determination

3.3.1. Hydrogel Polymerization

PEGDA was dissolved in HEPES-buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, pH = 7.4,) at concentrations of 10%, 20%, and 30% (w/v). 10 µl/ml of 300 mg/ml solution of 2,2-dimethyl-2-phenyl-acephenone (DMAP; TCI, Portland, OR) in N-vinylpyrrolidone (NVP; Sigma. St. Louis, MO) was added. 150 µl of each precursor solution was added to 48-well plates (Nalge Nunc, Rochester, NY), then photopolymerized for 2 min using a 365 nm transilluminator (Spectroline, Westbury, NY) at intensity of ~ 9 mW/cm^2 for 2 min.

3.3.2. Swelling Studies and Hydrogel Characterization

PEGDA hydrogel discs were blot dried and weighed immediately after polymerization and then allowed to swell in PBS (Hyclone, Logan, UT) at room temperature for 24 h to reach equilibrium. The discs were then weighed again to determine the equilibrium swollen mass, M_s. The salt was removed from swollen hydrogel discs by soaking them in diH_2O at room temperature for another 24 h. The hydrogel discs were dried by lyophilization for at least 24 h to get the dry mass, M_d. A sample size of two was used. The equilibrium water content (q) was calculated using the following equations:

$$q = \left(\frac{M_s}{M_d} - 1 \right) \times 100\% \qquad (1)$$

The number average molecular weight between crosslinks, M_c can be determined

using the following equation [33]:

$$\frac{1}{M_c} = \frac{2}{M_n} - \frac{(\bar{v}/V_1)[\ln(1-v_{2,s}) + v_{2,s} + cv_{2,s}^2]}{v_{2,r}[(v_{2,s}/v_{2,r})^{1/3} - \tfrac{1}{2}(v_{2,s}/v_{2,r})]} \quad (2)$$

where $\bar{v}$ = 0.870 cm^3/g, the specific volume of bulk PEG in the amorphous state, V_1 =18 cm^3/mol, the molar volume of water, $v_{2,s}$ and $v_{2,r}$ are the polymer volume faction of the hydrogel in the relaxed and swollen state, respectively, c = 0.426, the Flory-Huggins polymer-solvent interaction parameter, M_n = 10,000 Da, the number average molecular weight of PEGDA macromolecule. The average mesh size, z , of the hydrogels network was estimated using the following equation [33, 42, 43]:

$$z = lv_{2,s}^{-1/3}\left(\frac{3M_c}{M_r}\right)^{1/2} C_n^{1/2} \quad (3)$$

Where l = 1.50 Å, the backbone bond length, which was calculated by the weighted average of one-carbon-carbon bond and two carbon-oxygen bonds, M_r = 44 g/mol, the molecular weight of the PEG repeat unit, C_n = 4.0, the characteristic ration.

3.4. Cell Maintenance

Porcine vocal fold fibroblast cells (PVFF) were subcultured at 37˚C/5% CO_2 in Dulbecco's modified Eagle minimum essential medium (DMEM; Hyclone, Logan, UT) supplemented with 10% fetal bovine serum, 1 μg/ml bFGF, 50 mU/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine [30]. The experiments were conducted using cells at passages 6-8.

3.5. Cell Encapsulation

Precursor solution at concentrations of 10%, 20% and 30% (w/v) containing 2 mM of ACRL-PEG-RGDS (moles/swollen volume of hydrogels) and 10 μl/ml of a 300

mg/ml DMAP/NVP photoinitiator solution were sterilized by filtration (0.22 μm, PES membrane, Millipore, Bedford, MA). PVFF cells were trypsinized and resuspended in each precursor solution at a concentration of ~ 400,000 cells/ml (# cells/swollen volume of hydrogels). 2 ml of cell suspension was rapidly pipetted between two sterile clamped glass plates separated by 1.5 mm spacers and exposed to UV light (365 nm, ~ 9 mW/cm^2) for 2 min under constant turnover of the gel constructs. It has been shown that the longwave UV light used at similar intensities and exposure times for uniform photopolymerization results in minimal cell damage [44]. The prepared hydrogel constructs were transferred to a culture plate and incubated at 37˚C in a humidified environment with 5% CO_2. The hydrogel constructs were kept from the gas-media interface and the bottom surface of the plate by polyethylene spacers. The cell-encapsulated hydrogel constructs were cultured for 70 days in Dulbecco's modified Eagle minimum essential medium (DMEM, Hyclone, Logan, UT) supplemented with 10% fetal bovine serum, 50 mU/ml penicillin, 50 μg/ml streptomycin and 2 mM $_L$-glutamine. The medium was replaced every other day.

3.6. Compression Tests

The cell/hydrogel constructs were cultured for 2 days before compression test to ensure saturating hydration and cut into round discs of 11.5 mm in diameter. A sample size of 3 was used. The storage and loss moduli of each hydrogel disc were measured in compression mode using a dynamic mechanical analyzer (DMAQ800, TA Instruments, New Castle, DE). The bottom plate was clamped rigidly while the top plate was connected to a motor that enabled oscillation of the plate. Silicone oil (Fluka, Milwaukee, WI) was applied around the gel disc to slow down gel dehydration.

A dynamic strain sweep was performed at a frequency of 1 Hz at room temperature. The applied strain ranged from 1 μm to 50 μm. The storage modulus (E') and loss modulus (E") versus amplification were obtained. This information was used to ensure the frequency sweep tests were performed in the linear viscoelastic region where the measured mechanical properties are strain independent. A frequency sweep at a cyclic stain of 10 μm and preload force of 0.01 N was then performed over a range of 1 - 100 Hz, and graphs of the storage modulus (E') and loss modulus (E") versus frequency were obtained. These parameters are related by the following equations [45]:

$$E = E' + iE''$$
$$\tan d = E''/E' \quad (4)$$

where E is the dynamic modulus, $\tan d$ is loss tangent (damping ratio). The storage modulus represents elasticity of hydrogel, which means the capability of hydrogel to store mechanical energy and resist deformation. The loss modulus represents viscosity of hydrogel, a measurement of hydrogel's ability to resist shear flow.

3.7. Biochemical Assays

3.7.1. Hydrolysis of Samples

Samples were harvested at day 3, day 30 and day 70 of culture then transferred to screw-cap vials, weighed, flash-frozen in liquid nitrogen, and stored at -80ºC. Hydrogel samples were digested in 1 ml 0.1 N NaOH per 0.2 g hydrogel wet weight for 72 h at 37˚C. Digested hydrogel solutions were centrifuged (10,000 x g for 10 min), and a portion of each digest was taken for DNA and GAG quantification. Remaining solution was digested at 90 - 100˚C for 90 min to solubilize collagen but not elastin fibers. Hydrolyzed samples were centrifuged at 10,000 x g for 10 min to pellet

elastin. The supernatant was kept for collagen quantification. The elastin pellets were washed by diH2O at least four times and stored at -80˚C until use.

3.7.2. DNA Analysis

The DNA content in the gels was determined using a PicoGreen double-stranded DNA assay (Molecular Probes; Invitrogen, Carlsbad, CA) [30]. PEG hydrogels containing calf thymus DNA (Sigma, St. Louis, MO) were used as standards, which had experienced the same base hydrolysis conditions as the samples. Duplicate 40 μL aliquots of standards and samples were used in this assay such that all samples had the same concentrations of PEG. Samples were analyzed according to the manufacturer's instructions, using a Multi-Detection Microplate Reader (BioTek, Winooski, VT) with excitation filter at 480 nm and emission filter at 520 nm. A sample size of 6 was used. The cell number in each hydrogel was computed by using the conversion factor of 6.6 pg DNA per pig cell [30].

3.7.3. Sulfated GAGs Analysis

Sulfated GAGs production was measured with the Blyscan assay kit (Biocolor, Newtownabbey, Northern Ireland) [46]. This assay is based on the complex formation of dimethylmethylene blue with sulfated GAGs, a complex with an absorbance maximum at 525 nm. PEG hydrogels containing chondroitin sulfate B sodium salt (C3788; Sigma, St. Louis, MO) and which had experienced the same base hydrolysis conditions as the samples, were used as standards. Duplicate 80 μL aliquots of standards and samples were used such that all specimens had the same concentrations of PEG. The samples were neutralized with 10 N HCl (Fisher Scientific, Fair Lawn, NJ) and then mixed with 120 μL Blyscan Dye reagent. The resultant solution was measured immediately without delay using a Multi-Detection Microplate Reader at 525

nm. A sample size of 3 was used. The measured sulfated GAG content was expressed as micrograms of sulfated GAG per cell.

3.7.4. Collagen Content Measured by Hydroxyproline Assay

The hydroxyproline content, which is an indirect indicator of collagen, was determined by the dimethylbenzaldehyde (DMBA) assay. Aliquots of base-digests were combined with an equivalent volume of concentrated HCl (37%) to generate a 6N HCl solution and hydrolyzed 18 h at 110 ˚C in tubes with silicone-sealed gaskets. Acid hydrolyzed samples were dried by centrivap (Labconco. Kansas City, MI) and reconstituted in 100 µl diH2O. Samples were combined with 50 µL freshly prepared chloramine-T solution [88 mg chloramine-T in 0.625 ml isopropanol / 5 ml PH 6 buffer (2.24% Sodium Hydroxide, 3.2% Citric Acid Monohydrate, 8% Sodium Acetate Trihydate, 0.8% Acetic Acid Glacial, 20% Isopropanol; pH = 6.0)] and incubated for 15 min at room temperature. Samples were then mixed with 50 µL *p*-Dimethylbenzaldehyde reagent (1.25 g *p*-Dimethylbenzaldehyde in 5 ml Isopropanol, 2.25 ml Perchloric acid) and incubated at 37˚C for 30 min. The absorbance of samples at 550 nm was read with Multi-Detection Microplate Reader. PEG hydrogels containing L-4-hydroxyproline (Fluka, Milwaukee, WI) and which had experienced the same hydrolysis conditions as the samples, were used as standards. A sample size of 3 was used. Total collagen content was estimated from measured grams of hydroxyproline by dividing by 0.13, a conversion factor for collagen type I that is highly consistent across mammalian species [47]. Total collagen levels were expressed relative to the total cell density in the hydrogels.

3.7.5. Elastin Content Measured by Ninhydrin Assay

Elastin content was determined using the ninhydrin assay [48]. Elastin pellets were

digested in 6 N HCl at 110 ˚C for 18 h in tubes with silicone-sealed gaskets. Samples were then dried by centrivap and reconstituted in 220 μl 0.1 M sodium citrate buffer (pH = 5.0). An equivalent volume of ninhydrin reagent (Sigma, St. Louis, MO) was added to the samples and samples were boiled for 15 min. After cooling to room temperature, the absorbance of samples was read using a Multi-Detection Microplate Reader at 570 nm. A sample size of 3 was used. Hydrolyzed elastin (MP Biochemicals, Solon, Ohio) was used as the standard. The measured elastin content was expressed as micrograms of elastin per cell.

3.8. Histological Analysis and Immunohistochemistry

Hydrogel constructs were fixed with 10% formalin for 30 min, embedded in Tissue-Tek embedding media (Sakura Finetek, Torrance, CA) and sectioned at a thickness of 35 μm using a cryostat (Histotronix, Omaha, NE). Following the standard histological staining procedures, the cryosections were stained with toluidine blue solution (0.0714% toluidine blue (Fluka, Milwaukee, WI), 0.0714% pyronin Y (Fluka, Milwaukee, WI), and 0.143% borax (Fisher Scientific, Fair Lawn, NJ)) for 6 min to detect sulfated GAG. The collagen fibers were detected by staining with Picrosirius red solution (50 mg Sirius red F3B in 50 mL Saturated aqueous solution of picric acid) for 1 h. Stained sections were imaged using an Axiovert A200 microscope (Zeiss, Germany).

Sections for immunohistochemical staining were treated with 10% non-immune goat serum for 30 min. After treatment with goat serum, specimens were incubated at room temperature with primary antibody for elastin (mouse, Sigma, St. Louis, MO), collagen type I (rabbit, Rockland, Gibbertsville, PA), smooth muscle α-actin (mouse,

NeoMarkers, Fremont, CA) and vimentin (mouse, MS-129, NeoMarkers, Fremont, CA), then followed by the treatment with peroxidase (Biocare Medical, Concord, CA) for 30 min. The sections were treated with appropriate secondary antibody followed by streptavidin-biotin immunoenzymatic antigen detection kit (Zymed, South San Francisco, CA). Samples were stained without primary antibody as a negative control. Stained sections were imaged using an Axiovert A200 microscope (Zeiss, Germany). To determine the cell phenotype, the ratio of vimentin-positive and SMα-actin-positive cells in the samples at day 70 were calculated by the number of vimentin-positive or SMα-actin-positive cells in an immunohistochemical staining section divided by the sum of the cells in the same section.

3.9 Statistical Analysis

All data were reported as means ± standard deviation. The statistical analysis was performed by one-way ANOVA and Tukey's post hoc test using SigmaStat 3.5 (Systat Software, Inc. San Jose, CA). $p < 0.05$ was considered to be significantly different.

4. RESULTS

The results from the experiments described in Materials and Methods are presented below.

4.1. Determination of Mesh Size

The network microstructures of hydrogels with different PEGDA macromer concentrations were characterized by swelling studies. The polymer equilibrium water content (q), the number average molecular weight between crosslinks (M_c), and mesh size (z) were tabulated (Table 3) from these swelling ratios and the relationships detailed in PEG hydrogel mesh size and swelling. As shown in Table 3, although the equilibrium water content decreased slightly with increasing PEGDA macromer concentration, there was no statistically significant difference in z observed for different PEGDA hydrogels. This result is consistent with Cruise GM et al.'s results [40].

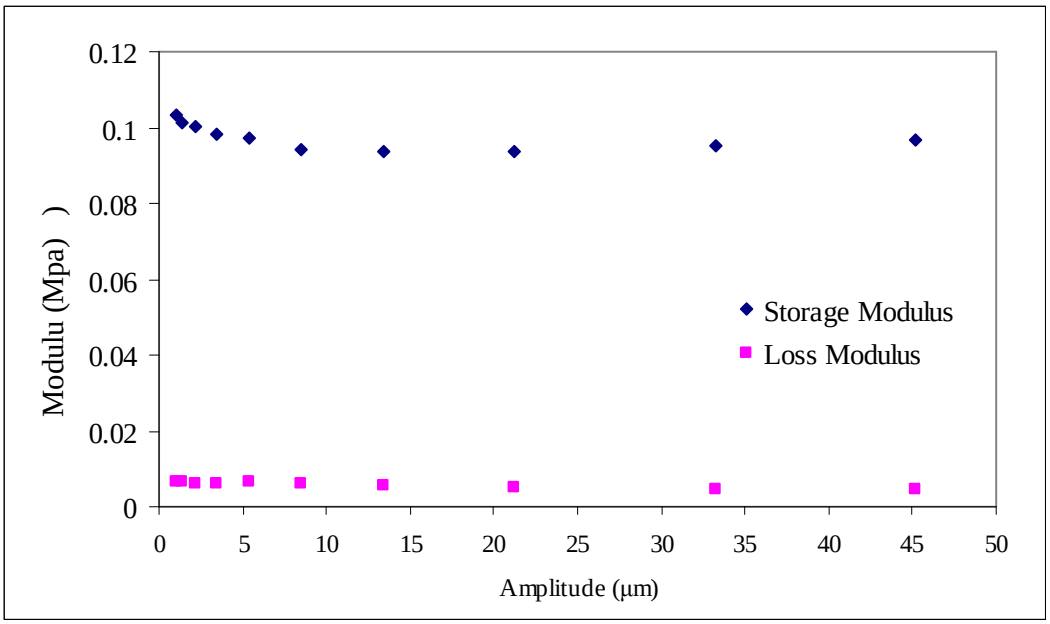
Table 3. Effect of PEGDA Concentration on the Network Structure of PEGDA Hydrogels

% PEGDA	q (%)	M_c (g/mol)	z (Å)
10%	92.10 ± 0.16	1220 ± 37	66.8 ± 1.4
20%	89.76 ± 0.05	1360 ± 9	63.6 ± 0.3
30%	88.54 ± 0.58	1407 ± 85	63.4 ± 3.0

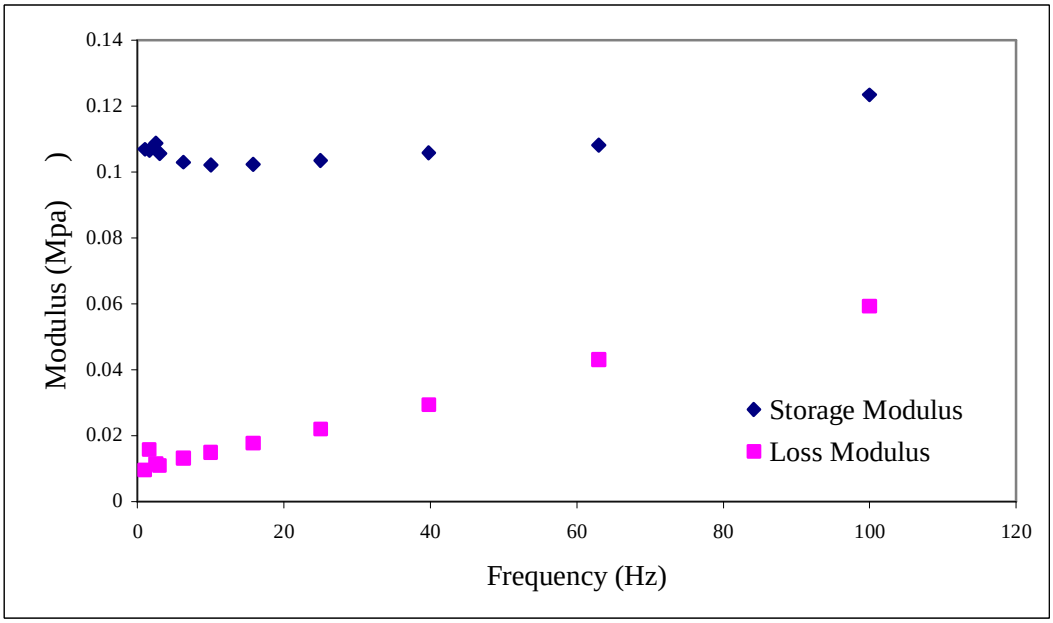
4.2. Mechanical Properties of PEGDA Hydrogels

In order to determine the initial mechanical environment experienced by PVFF, the

modulus of cell-hydrogel constructions were measured. Figure 5 (a) and (b) show the representative curves of storage modulus and loss modulus versus amplitude and frequency respectively. In Figure 5 (a), storage modulus and loss modulus remain constant after 10 μm, which means the measured moduli are strain independent. Figure 5 (b) shows storage modulus and loss modulus increased slowly with frequency, similar with previous observations of vocal fold tissue [5]. Since the major length changes of vocal folds during phonation occur at about 10 Hz and below [49], the storage modulus and loss modulus data of cell-hydrogel constructs at 1 Hz oscillatory frequency and 10μm strain from dynamic strain sweep measurement were tabulated (Table 4). Storage modulus of PEGDA hydrogels ($p < 0.002$) was increased with PEGDA macromer concentration in precursor solution. Only loss modulus of 10% PEGDA and 20% PEGDA gels showed significant difference ($p = 0.018$) and damping ratio ($p = 0.813$) of all the formulations were not significant different. In order to understand the viscoelasticity during high frequency during phonation, the mechanical of cell-hydrogel constructs at 40 Hz oscillatory frequency were determined by frequency sweep measurement (Table 4) ($p < 0.025$). It showed the similar trend as the data from strain sweep, with the storage modulus increasing with macromer concentration. The results of the mechanical testing combined with that of the swelling studies indicate that these hydrogel formulations can be used to probe the effects of mechanical properties on ECM synthesis uncoupled from variations in network structure.



(a)



(b)

Figure 5. (a) Storage modulus (E') and loss modulus (E'') of cell-hydrogel constructs (◆ Storage modulus, ■ Loss modulus) as a function of amplification for 30% PEGDA gels; (b) Storage modulus (E') and loss modulus (E'') of cell-hydrogel constructs as a function of frequency for 30% PEGDA.

Table 4. Mechanical Properties of PEGDA Hydrogels

	Stain sweep [a]			Frequency sweep [b]		
% PEGDA	E' [c] (kPa)	E'' [d] (kPa)	tan d [e]	E' [c] (kPa)	E'' [d] (kPa)	tan d [e]
10%	23.62 ± 1.70 *	2.54 ±1.48	0.108 ± 0.063	28.19 ± 0.12 *	12.84 ± 0.67	0.456 ± 0.024
20%	72.61 ± 4.76 *	12.25 ± 1.40	0.169 ± 0.022	87.54 ± 10.09 *	49.34 ± 13.89	0.564 ± 0.171
30%	94.25 ± 2.81 *	8.05 ±3.52	0.085 ±0.037	109.18 ± 3.25 *	35.44 ± 5.97	0.325 ± 0.056

a. Data was obtained at 10 μm at 1 Hz of strain sweeping measurement. (N = 3)
b. Data was obtained at 10 μm at 40 Hz of frequency sweeping measurement. (N = 3)
c. Storage modulus (elastic modulus)
d. Loss modulus (viscous modulus)
e. Damping ratio (the ratio of loss modulus to storage modulus)
* $p < 0.05$

4.3. Cell Density

Cell density in each hydrogel was assessed by determining hydrogel DNA levels. These measures were then used to calculate ECM production in each construct on a per cell basis. The cell density decreased by approximately 50% in 20% gels ($p < 0.001$) and 30% gels ($p < 0.001$) from day 3 to day 30, while no significant change in cell density was observed in 10% gels ($p = 0.904$) (Figure 6). However, there was no further cell density reduction in the 20% gels ($p = 0.938$) and 30% gels ($p = 0.895$) from day 30 to day 70.

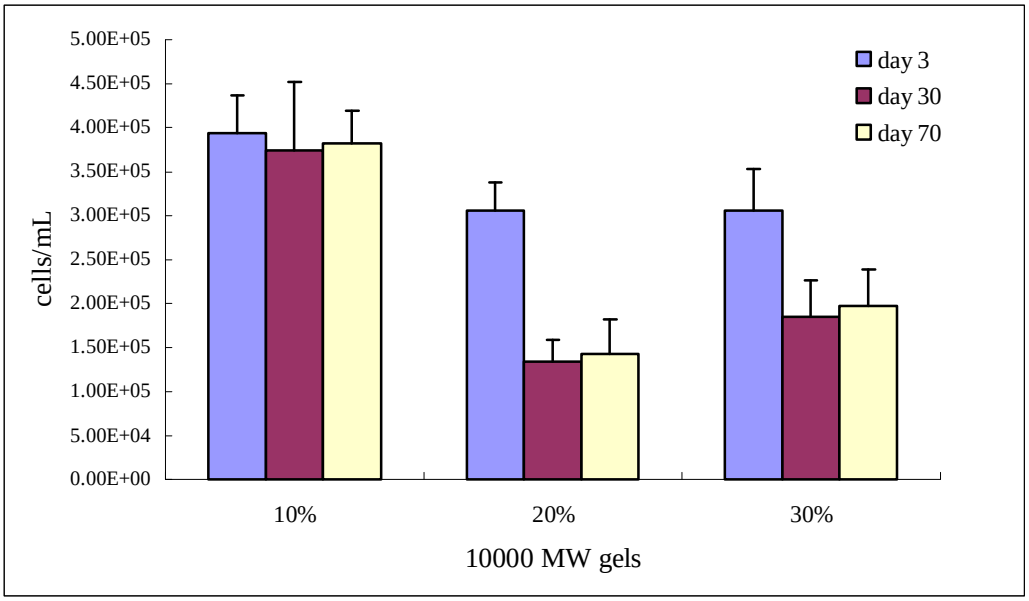


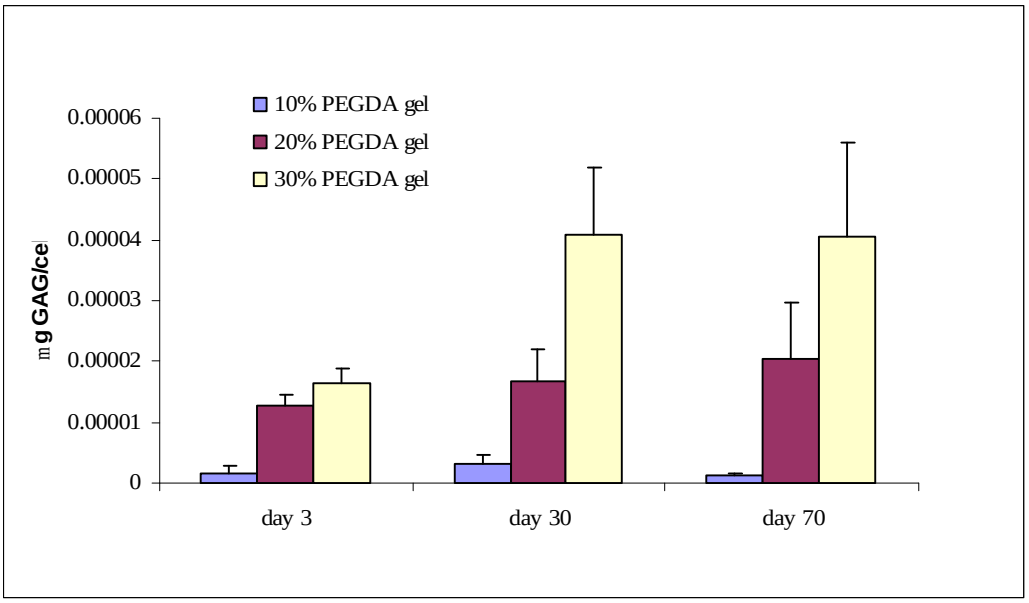
Figure 6. Cell density/ml of PVFF encapsulated in 10%, 20% and 30% PEGDA hydrogels at day 3, day 30 and day 70.

4.4. Matrix Synthesis in Hydrogels

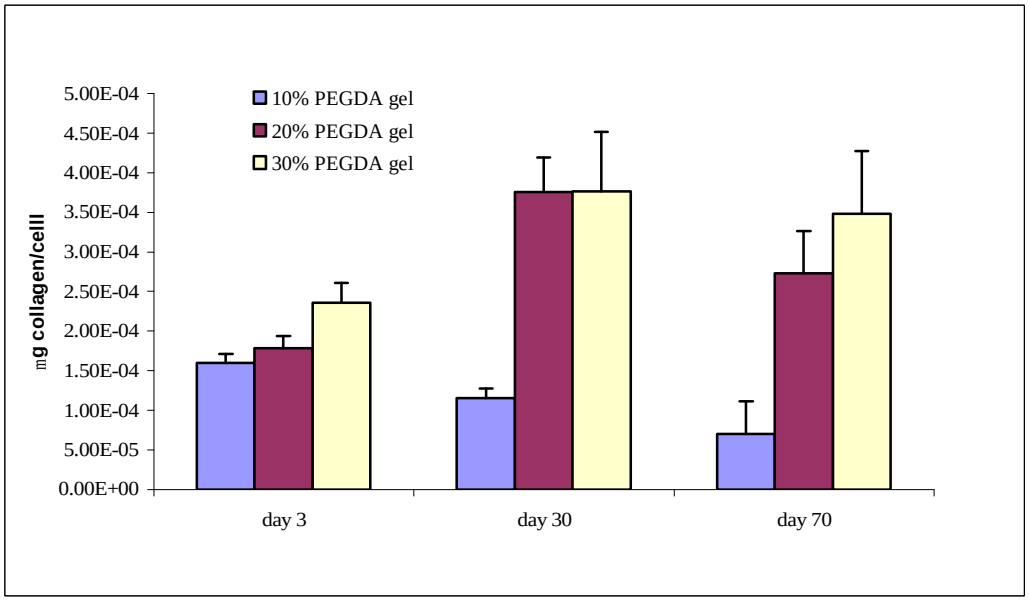
PEGDA constructs were harvested from culture at day 3, day 30 and day 70 for biochemical analysis. The measured GAGs/cell, collagen/cell and elastin/cell are

shown in Figures 7 (a), (b) and (c), respectively, as a function of time in culture and gel composition. Note that these data indicate that PVFF cells start to synthesize significant levels of new matrix within a short culture period (3 days). By day 3, PVFF in 10% gels produced significantly less GAGs/cell and collagen/cell than those in 30% gels ($p < 0.001$, $p = 0.026$ respectively) while PVFF in all gels produced similar amount of elastin ($p = 0.930$). By 30 days of static culture, however, the constructs generated from 20% PEGDA macromer solution promoted higher elastin/cell production than 10% and 30% PEGDA hydrogels ($p < 0.001$ and $p = 0.004$ respectively). Increased GAGs with increasing scaffold storage modulus from 10% to 30% PEGDA hydrogels was also noted both at 30 days and 70 days of culture ($p < 0.002$). Although collagen/cell levels in 20% and 30% PEGDA hydrogels were greater than those in 10% hydrogels after 30 days culture, no difference was observed between 20% and 30% gels. Interestingly, maximum ECM contents was observed in the hydrogels of intermediate storage modulus (20% PEGDA hydrogels) and total amount of major ECM components was lowest in the hydrogels of lowest storage modulus (10% PEGDA hydrogels) after day 30 ($p < 0.001$) and day 70 ($p < 0.001$).

However, the effect of culture time on cellular ECM production was small. More specifically, no significant difference was found in GAGs/cell production in 10%, 20% and 30% PEGDA hydrogels ($p = 0.088$, $p = 0.373$ and $p = 0.058$ respectively), in collagen/cell production in 30% PEGDA hydrogels ($p = 0.062$) and in elastin/cell production in 10% and 30% PEGDA hydrogels ($p = 0.540$ and $p = 0.159$ respectively) over the culture time. Furthermore, the total amount of major ECM components in 20% and 30% PEGDA hydrogels didn't increase significantly from day 30 to day 70 ($p = 0.758$ and $p = 0.169$ respectively).

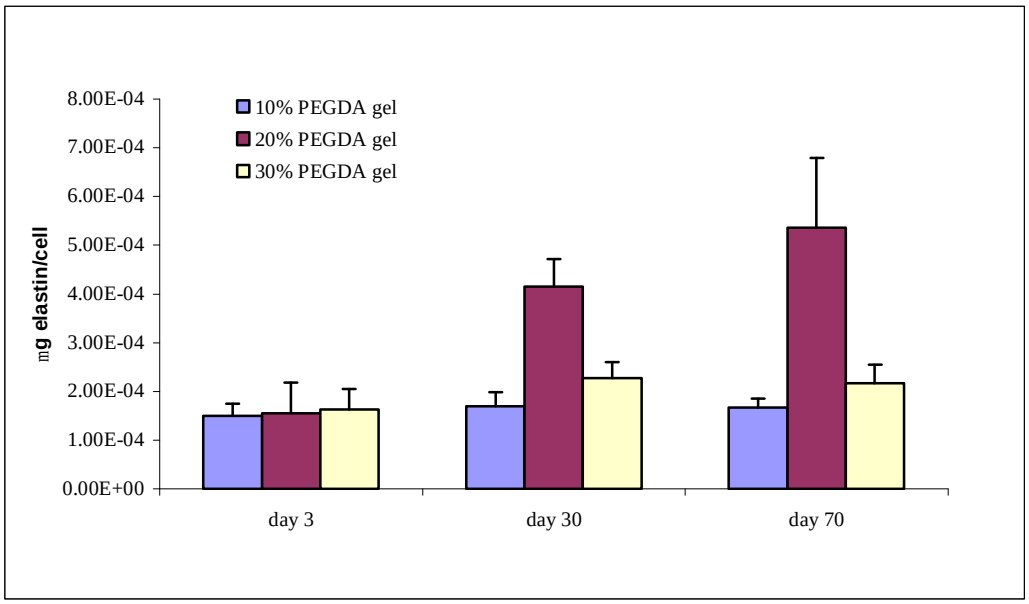


(a)

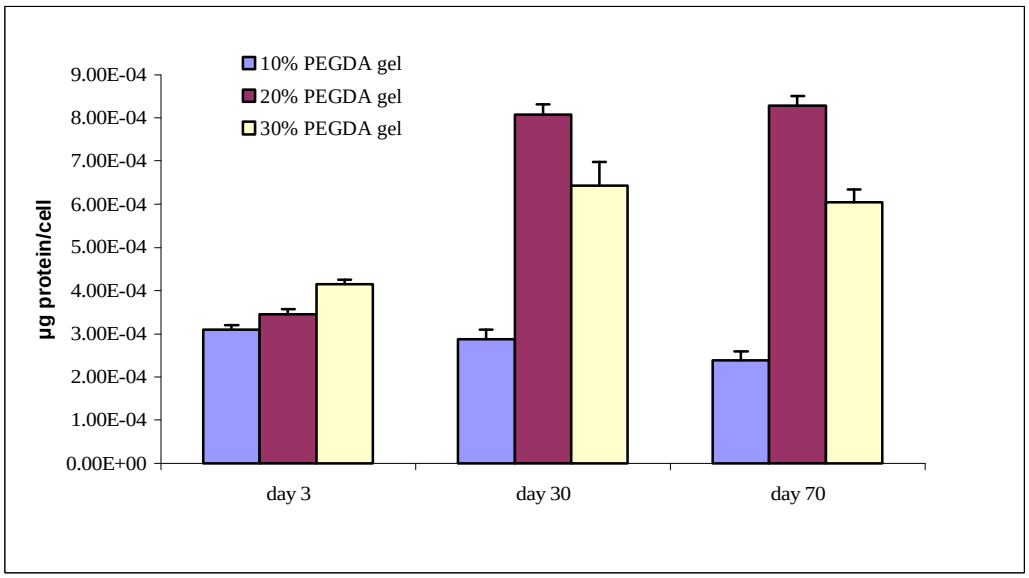


(b)

Figure 7. Cellular ECM production for 10%, 20% and 30% gels at day 3, day 30 and day 70: (a) sulfated GAGs, (b) collagen, (c) elastin, (d) the sum of sulfated GAGs, collagen and elastin per cell.



(c)

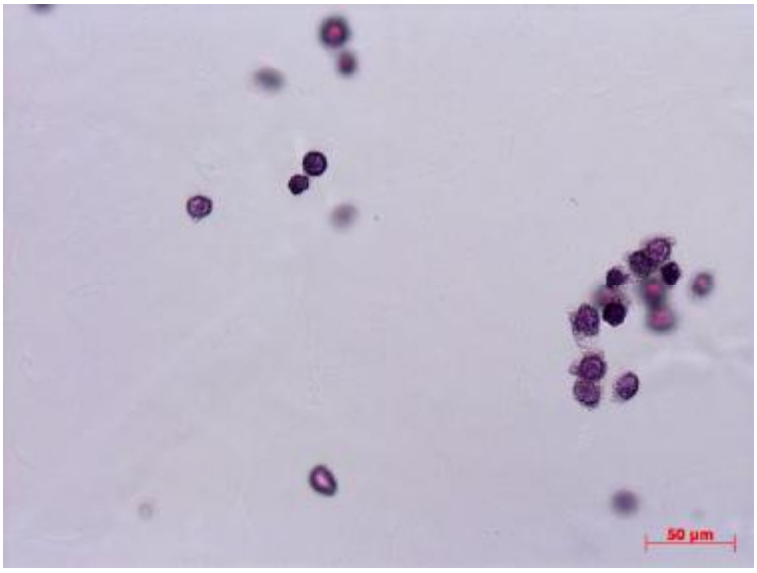


(d)

Figure 7. Continued.

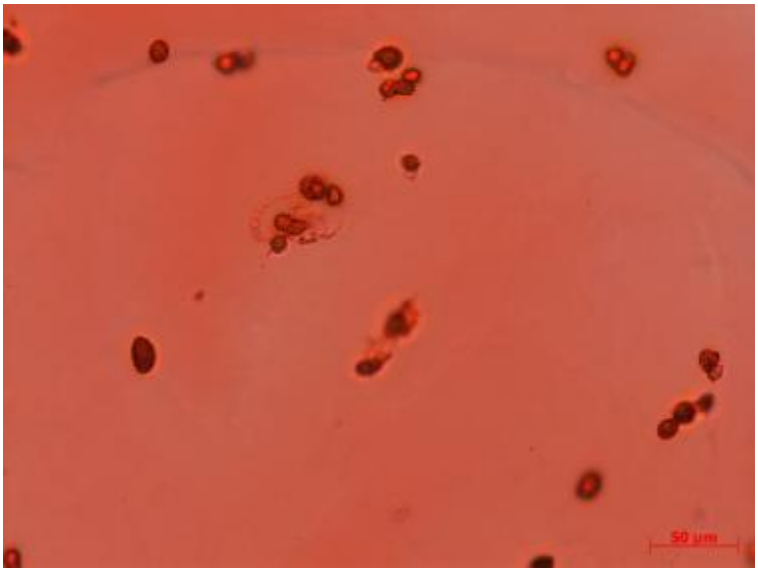
4.5 Histological Analysis

In addition to quantification of biochemical contents of PEGDA constructs, it is also important to learn the spatial distribution of ECM. The representative GAGs staining image, Figure 8 (a), revealed that PVFF had deposited GAGs only surrounding individual cells and no diffusion was observed. A similar pericellular distribution was observed in all 10%, 20% and 30% PEGDA gel constructs. Figure 8 (b) and (c) demonstrate that collagen and elastin were retained in the pericellular region and the region between cells.

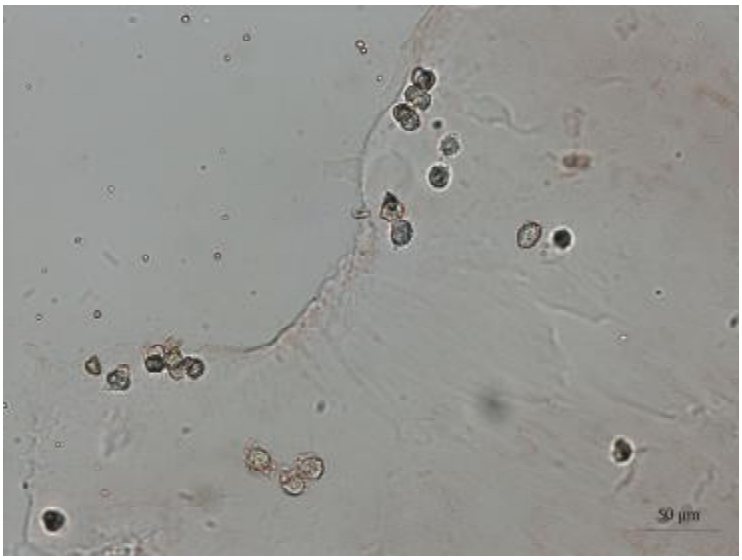


(a)

Figure 8. Representative images of major ECM protein distribution in PEGDA hydrogels after 70 days *in vitro*. These sections were from the 10% PEGDA hydrogels. (a) Collagen staining, picrosirius red straining collagen red; (b) GAGs staining, toluidine blue staining GAGs purple; (c) Elastin immunostaining, AEC chromogen yielding a deep red stain for elastin. The scale bar represents 50 µm.



(b)

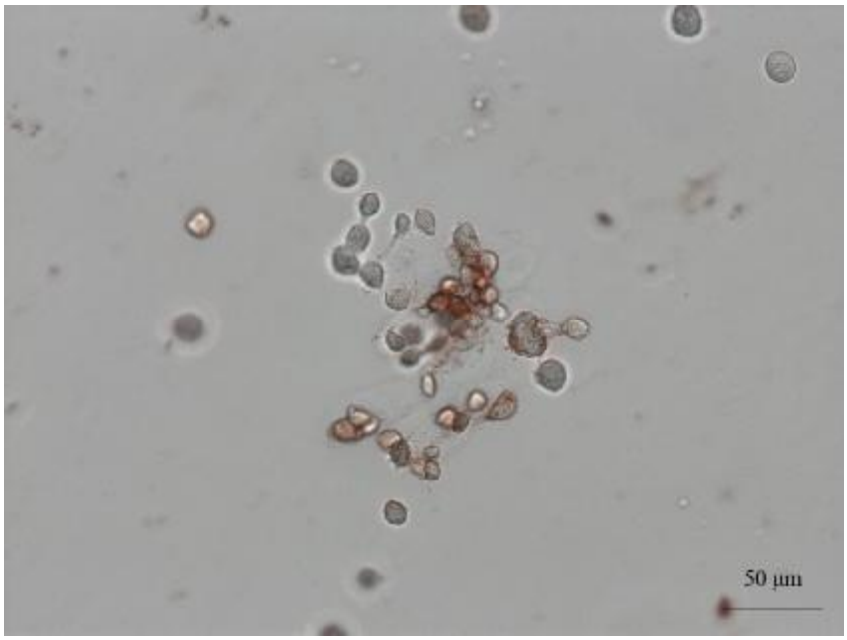


(c)

Figure 8. Continued.

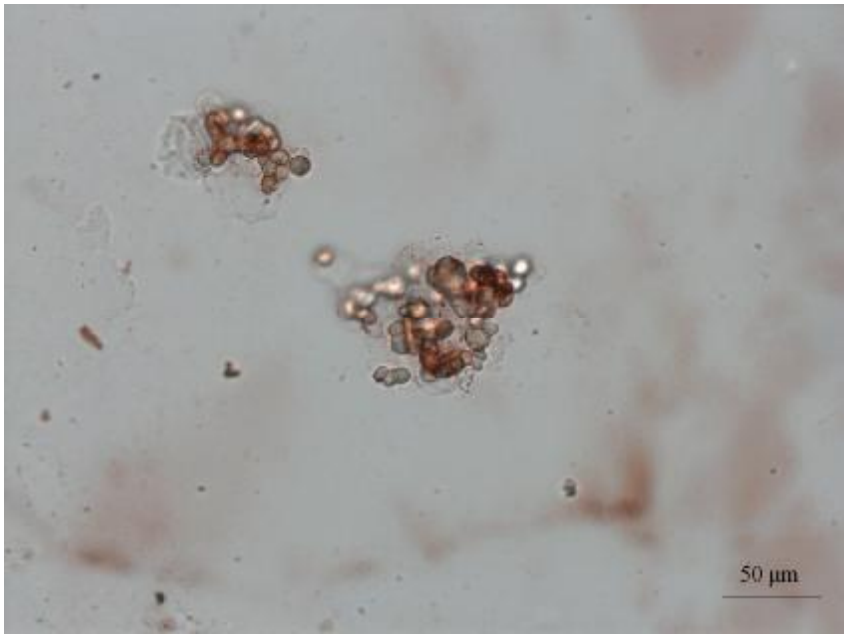
In addition to the total amount and distribution of collagen, the type of collagen produced is important. In lamina propria, the most abundant type of collagen is type I. The immunohistological analysis for collagen type I provide in-depth understanding in collagen distribution and organization. The representative images for collagen type I

immunohistological analysis in day 30, Figure 9 (a), (b) and (c), showed the organization of collagen was affected by the gel mechanical properties. In the 30% PEGDA hydrogel, with highest storage modulus, a collagen network structure between cells and some thick bundles of collagen were observed. Lowest staining density was found for collagen type I in 10% PEGDA hydrogel whereas no significant difference was observed between 20% and 30% PEGDA hydrogels staining, which is consistent with biochemical analysis.

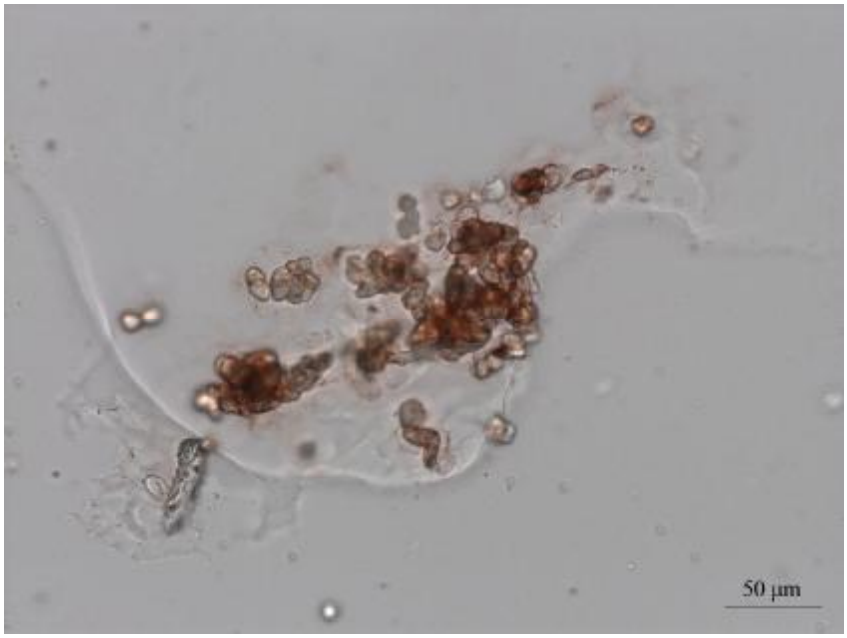


(a)

Figure 9. Immunohistological analysis of collagen type I after 30 days *in vitro*. AEC chromogen yields a deep red stain for collagen type I, (a) 10% PEGDA, (b) 20% PEGDA, (3) PEGDA. The scale bar represents 50 μm.



(b)



(c)

Figure 9. Continued.

Differentiation of PVFF in these gels is a major concern because the cells might change their phenotype into myofibroblasts in response to the mechanical microenvironment

around them. Immunohistochemistry was performed on these cell-hydrogel constructs after day 70 to assess cell phenotype, and the results are shown in Table 5. The vimentin amount increased with hydrogels' storage modulus ($p < 0.001$) and 10% PEGDA hydrogels showed fewest SMα-actin-positive cells. Since SMα-actin is only expressed in the myofibroblasts and myofibroblast also showed enhanced vimentin expression relative to fibroblasts [18], there appear to be more myofibroblasts in 20% and 30% PEGDA hydrogels.

Table 5. Vimentin and SMα-actin Ratio in PEGDA Hydrogels [b]

Sample	Vimentin ratio [a]	SMα-actin ratio [a]
10% PEGDA gels	0.371 ± 0.021 *	0.196 ± 0.000 *
20% PEGDA gels	0.520 ± 0.018 *	0.257 ± 0.012
30% PEGDA gels	0.591 ± 0.006 *	0.235 ± 0.003

a. The ratio of vimentin-positive and SMα-actin-positive cells in the samples at day 70 were calculated by the number of vimentin-positive or SMα-actin-positive cells in an immunohistochemical staining section divided by the sum of the cells in the same section.
b. N=2
* $p < 0.05$

5. DISCUSSION

Injectable hydrogels are highly desirable for vocal fold augmentation applications since they prevent further scarring induced by surgical procedures. PEG based hydrogels have been widely explored as encapsulation scaffolds suitable for tissue engineering due to their inherent biocompatibility and the fact that they are photopolymerizable materials, suitable for injectable implants without the need for invasive surgical intervention [30, 34 - 38]. In addition, PEGDA hydrogel is a cell nonadhesive substrate upon which cell-specific bioactivity can be written. They have been used as a model substrate to study 3-D migration [34]. The results presented here demonstrate the suitability of PEG as a model scaffold for examining the effects of mechanical properties of cell-hydrogel constructs on vocal fold fibroblasts' response. We hypothesized that the intrinsic mechanical properties of the microenvironment might influence cell ECM production. To carry out these experiments, we first needed to verify that mechanical properties and other scaffold features, like biochemistry and network structure can be tuned in an uncoupled manner, which implies that changing mechanical properties of PEGDA scaffolds does not simultaneously influence scaffold chemistry and network structure.

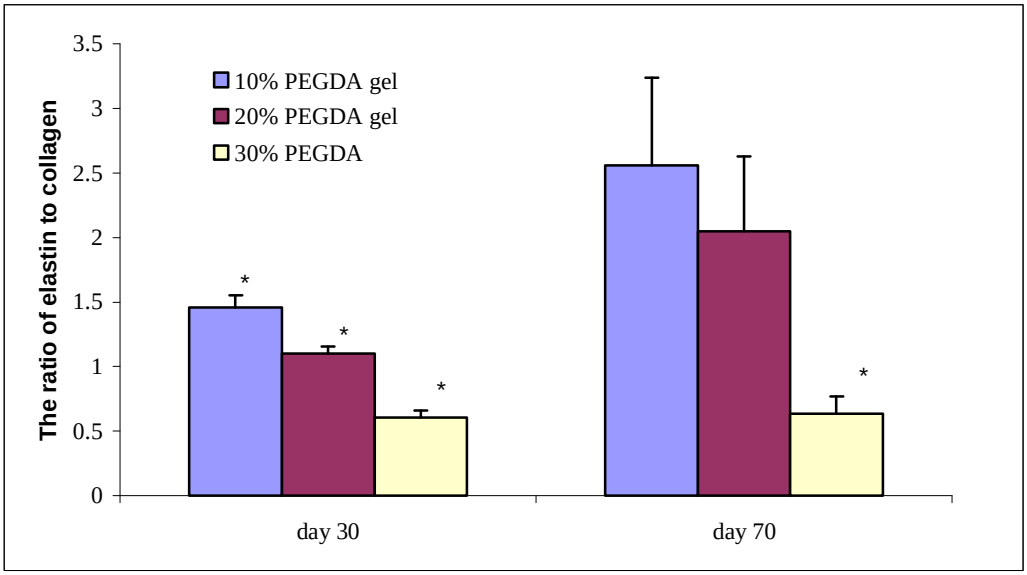
First of all, we characterized the bulk mechanical properties of PEG hydrogels to show that varying the macromer concentration in precursor solution enables systematic control over their mechanical properties (the storage modulus ranging from 23.62 ± 1.70 kPa to 94.25 ± 2.81 kPa), which agreed with previous studies on PEGDM hydrogels by Bryant et al.[33]. A higher storage modulus indicates a stiffer hydrogel. Furthermore, the swelling studies indicated there was no significant difference

between mesh size of hydrogel network for 10%, 20% and 30% PEGDA hydrogels. Thus, the gel mechanical properties were decoupled with mesh size successfully. In addition, since we modified the total RGDS amount/gel volume according to the swelling data, we could assume the RGDS density was held constant across all conditions. Any changes in cell behavior were then attributed to the change in PEGDA hydrogel mechanical properties.

From day 3 to day 30, the PVFF density in 20% and 30% PEGDA gel constructs was reduced while there was no significant cell density reduction in 10% PEGDA gel constructs. The decrease in cell density was observed in the PEGDA hydrogels since no adequate room was provided in non-degradable PEGDA hydrogels for cell to divide and migrate and the tightly cross-linked hydrogel (mesh size ~ 63 Å) hampered the extent of spreading of encapsulated cells. These results agree with the previous report that higher PEGDA composition in the gels comprises the cell viability over culture time [40].

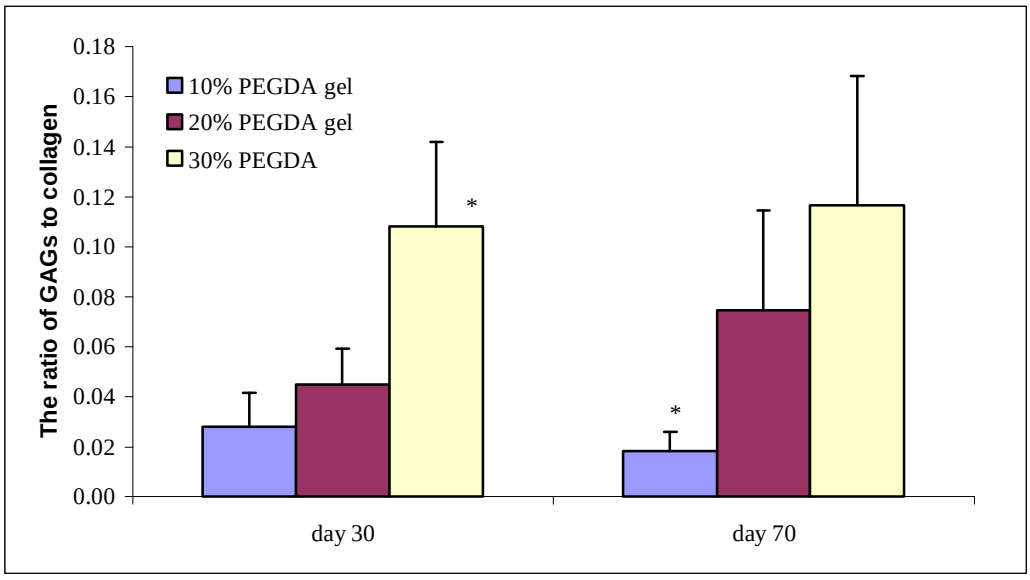
Biochemical data indicated the stiffer PEGDA hydrogels allowed the encapsulated PVFF to synthesize more collagen but that increasing the stiffness beyond a certain point could not increase collagen production. Thus, there appears to be an optimal stiffness at the present mesh size for PVFF to produce maximum elastin and collagen. To investigate the composition of ECM in different PEGDA hydrogels, we compared the ratio of elastin to collagen and GAG to collagen. As shown in Figure 10, the ratio of elastin to collagen decreased with an increase in gel stiffness ($p < 0.002$) and the higher ratio of GAGs to collagen was observed in 30% PEGDA hydrogels ($p < 0.01$). It indicates PVFF embedded in 30% PEGDA hydrogel synthesize more

GAG-rich ECM than other two kinds of PEGDA hydrogel while those in 10% PEGDA hydrogel produce more elastin-rich ECM. This result suggests the composition of ECM synthesis by cells can be altered by inherent mechanical properties of scaffold. Thus, it is possible to define optimal gel mechanical properties to control ECM composition according to different requirements for tissue engineering application.



(a)

Figure 10. ECM composition synthesized by PVFF encapsulated in 10%, 20% and 30% PEGDA hydrogels after day 30 and day 70, (a) the ratio of elastin to collagen, (b) ratio of GAGs to collagen. * representing the significant difference, $p < 0.05$.



(b)

Figure 10. Continued.

The data from day 30 to day 70 indicated culture time influenced GAGs/cell and elastin/cell production little. However, a decrease of collagen/cell production in 10% PEGDA hydrogels was observed from day 30 to day 70. The probable reason for this decrease may be explained by the well-known balance of ECM synthesis and degradation by matrix metalloproteinases (MMPs) [2]. ECM regulation is a precisely controlled process dependent not only on the amount of produced proteins, but also on activation or inhibition of MMPs by the tissue inhibitors of MMPs (TIMPs). This hypothesis is waiting to be tested by studying gene expression of collagen, MMP1 and TIMP4 using reverse transcriptase polymerase chain reaction.

The histological analysis showed GAGs, collagen and elastin to be present only around the cells in all three systems. The inhomogeneous GAGs, collagen and elastin distribution is due to tightly cross-linked hydrogel network. It is estimated the size of a small sized GAGs aggregate is approximately 90 Å in width and the size

of collagen type I fiber is 20 nm in average and elastin microfibrils can range from 110 – 130 nm in diameter [33, 50, 51]. The mesh size of PEGDA hydrogel investigated is ~ 63 Å, which is lower than the reported GAGs, collagen and elastin sizes. Therefore, it is expected that the newly synthesized collagen, elastin and GAGs are restricted to the pericellular regions. The immunohistological analysis for collagen suggested a dependence of collagen organization on gel stiffness. It was found collagen assembled into thick bundles in 30% PEGDA hydrogel, which is with highest storage modulus. This result is similar with the VFF's behavior in scarred tissue, in which fibroblasts undergo a phenotype change to myofibroblasts [2]. To test the hypothesis that the mechanical microenvironment around the cells might cause the phenotype change of vocal fold fibroblasts, the immunohistological analysis for vimentin and SMα-actin was performed. The primary result for cell phenotypes in the PEGDA hydrogels showed more myofibroblasts in 20% and 30% PEGDA hydrogels with higher stiffness. This change allows fibroblast to be more effective at ECM synthesis not only in maintaining the tissue but also repairing it. Clearly, to verify the hypothesis further, additional quantitative experiments are necessary to determine PVFF phenotype in PEGDA hydrogels.

6. CONCLUSION AND FUTURE RESEARCH

The aim of this study was to determine the effect of mechanical properties of PEGDA hydrogels on PVFF's behavior uncoupled from microstructural and biochemical property alterations. We demonstrated the gel mechanical properties were decoupled from mesh size of polymer network by varying the PEGDA macromer concentration. The biochemical analysis data suggested that increasing GAGs/cell and collagen/cell production was observed from 10% PEGDA hydrogels with lowest storage modulus to 20% PEGDA hydrogels. However, collagen/cell production did not change significantly with the further increase in storage modulus seen from the 20% to the 30% gels. Elastin/cell production is highest in 20% PEGDA hydrogel with intermediate storage modulus. In addition, the organization of collagen molecules in hydrogels and cell phenotype appeared to be influenced by the inherent mechanical properties of PEGDA hydrogels. Thus, the mechanical microenvironment around cell can control the composition of newly synthesized ECM and cell phenotypes. This PEGDA platform for investigating cell responses to specific scaffold property changes should allow optimal scaffold property design based on the requirements for clinical outcome of lamina propria engineering.

The PEGDA hydrogel model validated herein can be used to systematically study the complex interactions between scaffold features and cell response. The following scaffold features on VFF behavior can be investigated by this model:

1) Mesh size of the hydrogel network. Our previous study has shown 20% MW 10,000 PEGDA gel has the similar mechanical properties with 30% MW 20,000 PEGDA gel although they have different mesh size.

2) Biochemical properties. ECM components, like collagen, fibronectin, hyaluronic acid provide different biochemical stimuli to VFF. Many different types of ligands and molecules, derived from ECM components, can be covalently immobilized into a cell nonadhesive PEGDA hydrogel during photopolymerization to investigate single biochemical properties impacts on cell's response [34, 38].
3) Degradation rate. Biodegradable or bioerodible PEG hydrogels have been developed when hydrolytically degradable polymer segments or proteolytically degradable peptides were co-polymerized with PEG as a BAB block copolymers and then terminated with acrylate groups. By appropriately selecting the degradable segments, different hydrogel degradation rates can be achieved [33, 38].

These results from above proposed research on the effects of isolated scaffold features on VFF response will be important for rational scaffold design or implant material screening and will eventually benefit vocal fold restoration.

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APPENDIX

A.1. Water content

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	2	0	92.097	0.16	0.113
Sample-20%	2	0	89.761	0.0531	0.0376
Sample-30%	2	0	88.543	0.577	0.408

Source of Variation	DF	SS	MS	F	P
Between Groups	2	13.05	6.525	54.097	0.004
Residual	3	0.362	0.121		
Total	5	13.412			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
10% vs. 30%	3.554	3	14.474	0.004	Yes
10% vs. 20%	2.336	3	9.512	0.014	Yes
20% vs. 30%	1.219	3	4.962	0.078	No

A.2. Molecular weight between crosslinks

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	2	0	1220	37	26.163
Sample-20%	2	0	1360	9	6.364
Sample-30%	2	0	1407	85	60.104

Source of Variation	DF	SS	MS	F	P
Between Groups	2	37852	18926	6.545	0.081
Residual	3	8675	2891.667		
Total	5	46527			

A.3. Mesh size

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	2	0	66.805	1.427	1.009
Sample-20%	2	0	63.569	0.324	0.229
Sample-30%	2	0	63.403	2.981	2.108

Source of Variation	DF	SS	MS	F	P
Between Groups	2	14.715	7.357	2.002	0.28
Residual	3	11.024	3.675		
Total	5	25.739			

A.4. Mechanical properties

A.4.1. Storage modulus-strain sweep

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	2	0	23.615	1.704	1.205
Sample-20%	3	0	72.613	4.76	2.748
Sample-30%	3	0	94.25	2.806	1.62

Source of Variation	DF	SS	MS	F	P
Between Groups	2	6069.234	3034.617	237.231	<0.001
Residual	5	63.959	12.792		
Total	7	6133.193			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
30% vs. 10%	70.635	3	30.596	<0.001	Yes
30% vs. 20%	21.637	3	10.478	0.002	Yes
20% vs. 10%	48.998	3	21.224	<0.001	Yes

A.4.2. Loss modulus-strain sweep

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	2	0	2.543	1.478	1.045
Sample-20%	3	0	12.256	1.403	0.81
Sample-30%	3	0	8.051	3.522	2.034

Source of Variation	DF	SS	MS	F	P
Between Groups	2	113.404	56.702	9.164	0.021
Residual	5	30.936	6.187		
Total	7	144.34			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
20% vs. 10%	9.713	3	6.049	0.018	Yes
20% vs. 30%	4.206	3	2.929	0.191	No
30% vs. 10%	5.507	3	3.43	0.126	No

A.4.3. tan *d -strain sweep*

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	2	0	0.108	0.0631	0.0446
Sample-20%	3	0	0.169	0.0223	0.0129
Sample-30%	3	0	0.0854	0.0375	0.0216

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0.011	0.0055	3.534	0.111
Residual	5	0.00778	0.00156		
Total	7	0.0188			

A.4.4. Storage modulus-frequency sweep

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	2	0	28.193	0.12	0.0845
Sample-20%	3	0	87.541	10.088	5.824
Sample-30%	3	0	109.178	3.246	1.874

Source of Variation	DF	SS	MS	F	P
Between Groups	2	8087.22	4043.61	90.006	<0.001
Residual	5	224.629	44.926		
Total	7	8311.849			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
30% vs. 10%	80.985	3	18.718	<0.001	Yes
30% vs. 20%	21.637	3	5.591	0.025	Yes
20% vs. 10%	59.348	3	13.717	<0.001	Yes

A.4.5. Loss modulus-frequency sweep

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	2	0	12.845	0.675	0.477
Sample-20%	3	0	49.345	13.89	8.019
Sample-30%	3	0	35.443	5.974	3.449

Source of Variation	DF	SS	MS	F	P
Between Groups	2	1599.612	799.806	8.738	0.023
Residual	5	457.682	91.536		
Total	7	2057.294			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
20% vs. 10%	36.5	3	5.91	0.02	Yes
20% vs. 30%	13.902	3	2.517	0.268	No
30% vs. 10%	22.598	3	3.659	0.105	No

A.4.6. tan *d -frequency sweep*

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	2	0	0.456	0.024	0.017
Sample-20%	3	0	0.564	0.171	0.099
Sample-30%	3	0	0.325	0.0556	0.0321

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0.0859	0.043	3.277	0.123
Residual	5	0.0655	0.0131		
Total	7	0.151			

A.5. Cell density

A.5.1. Cell density in day 3

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	3	0	393865.749	42482.11	24527.06
Sample-20%	3	0	306287.63	30872.04	17823.98
Sample-30%	3	0	306305.73	46676.68	26948.79

Source of Variation	DF	SS	MS	F	P
Between Groups	2	15336684331	7668342165	4.66	0.06
Residual	6	9873049638	1645508273		
Total	8	25209733969			

A.5.2. Cell density in day 30

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	6	0	374210.9	78019	31851.1
Sample-20%	6	0	134178.8	25161.8	10272.3
Sample-30%	6	0	185241	41824.5	17074.8

Source of Variation	DF	SS	MS	F	P
Between Groups	2	1.92E+11	9.59E+10	33.981	<0.001
Residual	15	4.23E+10	2.82E+09		
Total	17	2.34E+11			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Means	p	q	P	P<0.050
10% vs. 20%	240032	3	11.066	<0.001	Yes
10% vs. 30%	188970	3	8.712	<0.001	Yes
30% vs. 20%	51062.1	3	2.354	0.251	No

A.5.3. Cell density in day 70

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	4	0	381531.116	37912.9	18956.45
Sample-20%	4	0	142837.287	39479.63	19739.82
Sample-30%	4	0	197641.454	41271.71	20635.86

Source of Variation	DF	SS	MS	F	P
Between Groups	2	1.25058E+11	62529099008	39.917	<0.001
Residual	9	14098152377	1566461375		
Total	11	1.39156E+11			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
10% vs. 20%	238693.8	3	12.062	<0.001	Yes
10% vs. 30%	183889.7	3	9.292	<0.001	Yes
30% vs. 20%	54804.17	3	2.769	0.178	No

A.5.4. Cell density in 10% PEGDA

Group Name	N	Missing	Mean	Std Dev	SEM
Day 3	3	0	393865.749	42482.11	24527.06
Day 30	6	0	374210.917	78019.03	31851.14
Day 70	4	0	381531.116	37912.9	18956.45

Source of Variation	DF	SS	MS	F	P
Between Groups	2	774260753.8	387130376.9	0.101	0.905
Residual	10	38356467137	3835646714		
Total	12	39130727891			

A.5.5. Cell density in 20% PEGDA

Group Name	N	Missing	Mean	Std Dev	SEM
Day 3	3	0	306287.63	30872.04	17823.98
Day 30	6	0	134178.831	25161.84	10272.28
Day 70	4	0	142837.287	39479.63	19739.82

Source of Variation	DF	SS	MS	F	P
Between Groups	2	65813640382	32906820191	33.759	<0.001
Residual	10	9747680798	974768079.8		
Total	12	75561321181			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
Day3 vs. Day30	172108.8	3	11.025	<0.001	Yes
Day3 vs. Day70	163450.3	3	9.694	<0.001	Yes
Day70 vs. Day30	8658.455	3	0.608	0.904	No

A.5.6. Cell density in 30% PEGDA

Group Name	N	Missing	Mean	Std Dev	SEM
Day 3	3	0	306305.73	46676.68	26948.79
Day 30	6	0	185240.959	41824.53	17074.79
Day 70	4	0	197641.454	41271.71	20635.86

Source of Variation	DF	SS	MS	F	P
Between Groups	2	31477373045	15738686522	8.641	0.007
Residual	10	18213945869	1821394587		
Total	12	49691318913			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
Day3 vs. Day30	121064.8	3	5.673	0.006	Yes
Day3 vs. Day70	108664.3	3	4.715	0.019	Yes
Day70 vs. Day30	12400.5	3	0.637	0.896	No

A.6. GAGs

A.6.1. GAGs in day 3

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	3	0	1.43E-06	1.24E-06	0.000000719
Sample-20%	3	0	1.27E-05	1.99E-06	0.00000115
Sample-30%	3	0	1.64E-05	2.58E-06	0.00000149

Source of Variatio	DF	SS	MS	F	P
Between Groups	2	3.64E-10	1.82E-10	44.874	<0.001
Residual	6	2.44E-11	4.06E-12		
Total	8	3.89E-10			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
30% vs. 10%	0.000015	3	12.86	<0.001	Yes
30% vs. 20%	3.7E-06	3	3.178	0.141	No
20% vs. 10%	1.13E-05	3	9.683	0.001	Yes

A.6.2. GAGs in day 30

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	3	0	3.25E-06	1.47E-06	8.46E-07
Sample-20%	3	0	1.68E-05	5.02E-06	2.9E-06
Sample-30%	3	0	4.08E-05	1.13E-05	6.53E-06

Source of Variatio	DF	SS	MS	F	P
Between Groups	2	2.16E-09	1.08E-09	20.908	0.002
Residual	6	3.1E-10	5.18E-11		
Total	8	2.47E-09			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
30% vs. 10%	3.75E-05	3	9.032	0.002	Yes
30% vs. 20%	2.39E-05	3	5.758	0.016	Yes
20% vs. 10%	1.36E-05	3	3.273	0.129	No

A.6.3. GAGs in day 70

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	4	0	1.29E-06	2.5E-07	1.25E-07
Sample-20%	4	0	2.04E-05	9.26E-06	4.63E-06
Sample-30%	4	0	4.06E-05	1.54E-05	7.7E-06

Source of Variatio	DF	SS	MS	F	P
Between Groups	2	3.08E-09	1.54E-09	14.312	0.002
Residual	9	9.7E-10	1.08E-10		
Total	11	4.05E-09			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
30% vs. 10%	3.93E-05	3	7.565	0.001	Yes
30% vs. 20%	2.01E-05	3	3.879	0.054	No
20% vs. 10%	1.91E-05	3	3.687	0.067	No

A.6.4. GAGs in 10% PEGDA

Group Name	N	Missing	Mean	Std Dev	SEM
Day 3	3	0	0.00000143	1.24E-06	7.19E-07
Day 30	3	0	0.00000325	1.47E-06	8.46E-07
Day 70	4	0	0.00000129	2.5E-07	1.25E-07

Source of Variatio	DF	SS	MS	F	P
Between Groups	2	7.58E-12	3.79E-12	3.5	0.088
Residual	7	7.58E-12	1.08E-12		
Total	9	1.52E-11			

A.6.5. GAGs in 20% PEGDA

Group Name	N	Missing	Mean	Std Dev	SEM
Day 3	3	0	0.0000127	1.99E-06	1.15E-06
Day 30	3	0	0.0000168	5.02E-06	2.9E-06
Day 70	4	0	0.0000204	9.26E-06	4.63E-06

Source of Variation	DF	SS	MS	F	P
Between Groups	2	1.03E-10	5.13E-11	1.139	0.373
Residual	7	3.16E-10	4.51E-11		
Total	9	4.18E-10			

A.6.6. GAGs in 30% PEGDA

Group Name	N	Missing	Mean	Std Dev	SEM
Day 3	3	0	0.0000164	2.58E-06	1.49E-06
Day 30	3	0	0.0000408	1.13E-05	6.53E-06
Day 70	4	0	0.0000406	1.54E-05	7.7E-06

Source of Variation	DF	SS	MS	F	P
Between Groups	2	1.24E-09	6.18E-10	4.406	0.058
Residual	7	9.82E-10	1.4E-10		
Total	9	2.22E-09			

A.7. Collagen

A.7.1. Collagen in day 3

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	3	0	0.00016	0.000011	6.35E-06
Sample-20%	3	0	0.000178	1.22E-05	7.05E-06
Sample-30%	3	0	0.000236	4.13E-05	2.39E-05

Source of Variation	DF	SS	MS	F	P
Between Groups	2	9.36E-09	4.68E-09	7.106	0.026
Residual	6	3.95E-09	6.59E-10		
Total	8	1.33E-08			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
30% vs. 10%	7.58E-05	3	5.113	0.026	Yes
30% vs. 20%	5.73E-05	3	3.866	0.076	No
20% vs. 10%	1.85E-05	3	1.247	0.671	No

A.7.2. Collagen in day 30

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	3	0	0.000115	1.57E-05	9.07E-06
Sample-20%	3	0	0.000376	4.38E-05	0.0000253
Sample-30%	3	0	0.000377	5.31E-05	0.0000306

Source of Variation	DF	SS	MS	F	P
Between Groups	2	1.36E-07	6.8E-08	40.943	<0.001
Residual	6	9.96E-09	1.66E-09		
Total	8	1.46E-07			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
30% vs. 10%	0.000261	3	11.102	<0.001	Yes
30% vs. 20%	9.24E-07	3	0.0393	1	No
20% vs. 10%	0.00026	3	11.063	<0.001	Yes

A.7.3. Collagen in day 70

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	4	0	7.03E-05	2.52E-05	0.0000126
Sample-20%	4	0	0.000273	7.54E-05	0.0000377
Sample-30%	4	0	0.000348	7.95E-05	0.0000397

Source of Variation	DF	SS	MS	F	P
Between Groups	2	1.65E-07	8.27E-08	19.633	<0.001
Residual	9	3.79E-08	4.21E-09		
Total	11	2.03E-07			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
30% vs. 10%	0.000278	3	8.563	<0.001	Yes
30% vs. 20%	7.48E-05	3	2.306	0.283	No
20% vs. 10%	0.000203	3	6.257	0.004	Yes

A.7.4. Collagen in 10% PEGDA

Group Name	N	Missing	Mean	Std Dev	SEM
Day 3	3	0	0.00016	0.000011	6.35E-06
Day 30	3	0	0.000115	1.57E-05	9.07E-06
Day 70	4	0	7.03E-05	2.52E-05	1.26E-05

Source of Variation	DF	SS	MS	F	P
Between Groups	2	1.39E-08	6.94E-09	18.452	0.002
Residual	7	2.63E-09	3.76E-10		
Total	9	1.65E-08			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
Day3 vs. Day70	8.97E-05	3	8.563	0.001	Yes
Day3 vs. Day30	4.46E-05	3	3.984	0.06	No
Day30 vs. Day70	4.51E-05	3	4.303	0.044	Yes

A.7.5. Collagen in 20% PEGDA

Group Name	N	Missing	Mean	Std Dev	SEM
Day 3	3	0	0.000178	1.22E-05	7.05E-06
Day 30	3	0	0.000376	4.38E-05	2.53E-05
Day 70	4	0	0.000273	7.54E-05	3.77E-05

Source of Variation	DF	SS	MS	F	P
Between Groups	2	5.84E-08	2.92E-08	9.642	0.01
Residual	7	2.12E-08	3.03E-09		
Total	9	7.95E-08			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
Day30 vs. Day3	0.000197	3	6.208	0.008	Yes
Day30 vs. Day70	0.000102	3	3.446	0.101	No
Day70 vs. Day3	9.48E-05	3	3.192	0.129	No

A.7.6. Collagen in 30% PEGDA

Group Name	N	Missing	Mean	Std Dev	SEM
Day 3	3	0	0.000236	4.13E-05	2.39E-05
Day 30	3	0	0.000377	5.31E-05	3.06E-05
Day 70	4	0	0.000348	7.95E-05	3.97E-05

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0.000000034	1.7E-08	4.247	0.062
Residual	7	0.000000028	4E-09		
Total	9	0.000000062			

A.8. Elastin

A.8.1. Elastin in day 3

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	3	0	0.000149	2.58E-05	1.49E-05
Sample-20%	3	0	0.000155	0.000063	3.64E-05
Sample-30%	3	0	0.000163	4.21E-05	2.43E-05

Source of Variation	DF	SS	MS	F	P
Between Groups	2	3.13E-10	1.57E-10	0.0733	0.93
Residual	6	1.28E-08	2.14E-09		
Total	8	1.31E-08			

A.8.2. Elastin in day 30

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	3	0	0.000169	0.000029	0.0000168
Sample-20%	3	0	0.000415	5.67E-05	0.0000327
Sample-30%	3	0	0.000227	0.000033	0.0000191

Source of Variation	DF	SS	MS	F	P
Between Groups	2	9.91E-08	4.96E-08	28.919	<0.001
Residual	6	1.03E-08	1.71E-09		
Total	8	1.09E-07			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
20% vs. 10%	0.000246	3	10.286	0.001	Yes
20% vs. 30%	0.000188	3	7.865	0.004	Yes
30% vs. 10%	5.79E-05	3	2.42	0.276	No

A.8.3. Elastin in day 70

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	4	0	0.000167	1.75E-05	8.76E-06
Sample-20%	4	0	0.000535	0.000143	0.0000715
Sample-30%	4	0	0.000216	0.000039	0.0000195

Source of Variation	DF	SS	MS	F	P
Between Groups	2	3.19E-07	1.6E-07	21.497	<0.001
Residual	9	6.69E-08	7.43E-09		
Total	11	3.86E-07			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
20% vs. 10%	0.000368	3	8.537	<0.001	Yes
20% vs. 30%	0.000319	3	7.403	0.002	Yes
30% vs. 10%	4.89E-05	3	1.134	0.711	No

A.8.4. Elastin in 10% PEGDA

Group Name	N	Missing	Mean	Std Dev	SEM
Day 3	3	0	0.000149	2.58E-05	1.49E-05
Day 30	3	0	0.000169	0.000029	1.68E-05
Day 70	4	0	0.000167	1.75E-05	8.76E-06

Source of Variation	DF	SS	MS	F	P
Between Groups	2	7.58E-10	3.79E-10	0.674	0.54
Residual	7	3.94E-09	5.63E-10		
Total	9	4.7E-09			

A.8.5. Elastin in 20% PEGDA

Group Name	N	Missing	Mean	Std Dev	SEM
Day 3	3	0	0.000155	0.000063	3.64E-05
Day 30	3	0	0.000415	5.67E-05	3.27E-05
Day 70	4	0	0.000535	0.000143	7.15E-05

Source of Variation	DF	SS	MS	F	P
Between Groups	2	2.52E-07	1.26E-07	11.658	0.006
Residual	7	7.58E-08	1.08E-08		
Total	9	3.28E-07			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
Day70 vs. Day3	0.000381	3	6.777	0.005	Yes
Day70 vs. Day30	0.00012	3	2.145	0.34	No
Day30 vs. Day3	0.00026	3	4.333	0.043	Yes

A.8.6. Elastin in 30% PEGDA

Group Name	N	Missing	Mean	Std Dev	SEM
Day 3	3	0	0.000163	4.21E-05	2.43E-05
Day 30	3	0	0.000227	0.000033	1.91E-05
Day 70	4	0	0.000216	0.000039	1.95E-05

Source of Variation	DF	SS	MS	F	P
Between Groups	2	7.1E-09	3.55E-09	2.418	0.159
Residual	7	1.03E-08	1.47E-09		
Total	9	1.74E-08			

A.9. Total ECM protein

A.9.1. Sum of ECM protein in day 3

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	3	0	0.000311	9.37E-06	5.41E-06
Sample-20%	3	0	0.000346	2.14E-05	0.0000124
Sample-30%	3	0	0.000416	1.97E-05	0.0000114

Source of Variatio	DF	SS	MS	F	P
Between Groups	2	1.71E-08	8.57E-09	27.572	<0.001
Residual	6	1.87E-09	3.11E-10		
Total	8	1.9E-08			

A.9.2. Sum of ECM proteins in day 30

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	3	0	0.000288	0.000011	6.36E-06
Sample-20%	3	0	0.000807	2.39E-05	0.0000138
Sample-30%	3	0	0.000644	2.12E-05	0.0000122

Source of Variatio	DF	SS	MS	F	P
Between Groups	2	4.24E-07	2.12E-07	556.715	<0.001
Residual	6	2.28E-09	3.81E-10		
Total	8	4.26E-07			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
20% vs. 10%	0.00052	3	46.137	<0.001	Yes
20% vs. 30%	0.000163	3	14.484	<0.001	Yes
30% vs. 10%	0.000357	3	31.653	<0.001	Yes

A.9.3. Sum of ECM proteins in day 70

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	3	0	0.000239	1.02E-05	0.0000059
Sample-20%	3	0	0.000829	0.000054	0.0000312
Sample-30%	3	0	0.000605	2.99E-05	0.0000173

Source of Variatio	DF	SS	MS	F	P
Between Groups	2	5.32E-07	2.66E-07	203.914	<0.001
Residual	6	7.83E-09	1.31E-09		
Total	8	5.4E-07			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
20% vs. 10%	0.00059	3	28.289	<0.001	Yes
20% vs. 30%	0.000224	3	10.744	<0.001	Yes
30% vs. 10%	0.000366	3	17.544	<0.001	Yes

A.9.4. Sum of ECM proteins in 10% PEGDA

Group Name	N	Missing	Mean	Std Dev	SEM
Day 3	3	0	0.000311	9.37E-06	0.00000541
Day 30	3	0	0.000288	0.000011	0.00000636
Day 70	4	0	0.000239	1.02E-05	0.00000511

Source of Variation	DF	SS	MS	F	P
Between Groups	2	9.46E-09	4.73E-09	45.264	<0.001
Residual	7	7.31E-10	1.04E-10		
Total	9	1.02E-08			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
Day3 vs. Day70	7.16E-05	3	12.965	<0.001	Yes
Day3 vs. Day30	2.29E-05	3	3.889	0.065	No
Day30 vs. Day70	4.86E-05	3	8.808	0.001	Yes

A.9.5. Sum of ECM proteins in 20% PEGDA

Group Name	N	Missing	Mean	Std Dev	SEM
Day 3	3	0	0.000346	2.14E-05	1.24E-05
Day 30	3	0	0.000807	2.39E-05	1.38E-05
Day 70	4	0	0.000829	0.000054	0.000027

Source of Variation	DF	SS	MS	F	P
Between Groups	2	4.73E-07	2.36E-07	153.069	<0.001
Residual	7	1.08E-08	1.54E-09		
Total	9	4.83E-07			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
Day70 vs. Day3	0.000483	3	22.776	<0.001	Yes
Day70 vs. Day30	2.17E-05	3	1.023	0.758	No
Day30 vs. Day3	0.000462	3	20.348	<0.001	Yes

A.9.6. Sum of ECM proteins in 30% PEGDA

Group Name	N	Missing	Mean	Std Dev	SEM
Day 3	3	0	0.000416	1.97E-05	1.14E-05
Day 30	3	0	0.000644	2.12E-05	1.22E-05
Day 70	4	0	0.000605	2.99E-05	0.000015

Source of Variation	DF	SS	MS	F	P
Between Groups	2	9.19E-08	4.59E-08	73.754	<0.001
Residual	7	4.36E-09	6.23E-10		
Total	9	9.63E-08			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
Day30 vs. Day3	0.000229	3	15.863	<0.001	Yes
Day30 vs. Day70	3.92E-05	3	2.912	0.169	No
Day70 vs. Day3	0.000189	3	14.046	<0.001	Yes

A.10. The ratio of elastin to collagen

A.10.1. Elastin/Collagen in day 30

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	3	0	1.462	0.0918	0.053
Sample-20%	3	0	1.103	0.055	0.0317
Sample-30%	3	0	0.604	0.0571	0.033

Source of Variation	DF	SS	MS	F	P
Between Groups	2	1.115	0.557	113.675	<0.001
Residual	6	0.0294	0.0049		
Total	8	1.144			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
10% vs. 30%	0.858	3	21.229	<0.001	Yes
10% vs. 20%	0.359	3	8.877	0.002	Yes
20% vs. 30%	0.499	3	12.352	<0.001	Yes

A.10.2. Elastin/Collagen in day 70

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	4	0	2.563	0.678	0.339
Sample-20%	4	0	2.047	0.586	0.293
Sample-30%	4	0	0.637	0.131	0.0655

Source of Variation	DF	SS	MS	F	P
Between Groups	2	7.945	3.972	14.55	0.002
Residual	9	2.457	0.273		
Total	11	10.402			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
10% vs. 30%	1.925	3	7.369	0.002	Yes
10% vs. 20%	0.516	3	1.975	0.383	No
20% vs. 30%	1.409	3	5.394	0.01	Yes

A.11. The ratio of GAGs to collagen

A.11.1. GAGs/Collagen in day 30

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	3	0	0.0281	0.0133	0.00766
Sample-20%	3	0	0.0448	0.0143	0.00828
Sample-30%	3	0	0.108	0.0337	0.0194

Source of Variatio	DF	SS	MS	F	P
Between Groups	2	0.0107	0.00536	10.597	0.011
Residual	6	0.00303	0.000506		
Total	8	0.0137			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
30% vs. 10%	0.0801	3	6.17	0.011	Yes
30% vs. 20%	0.0634	3	4.884	0.032	Yes
20% vs. 10%	0.0167	3	1.286	0.655	No

A.11.2. GAGs/Collagen in day 70

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	4	0	0.0183	0.00747	0.00374
Sample-20%	4	0	0.0747	0.0397	0.0198
Sample-30%	4	0	0.117	0.0516	0.0258

Source of Variatio	DF	SS	MS	F	P
Between Groups	2	0.0194	0.00971	6.778	0.016
Residual	9	0.0129	0.00143		
Total	11	0.0323			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
30% vs. 10%	0.0982	3	5.188	0.013	Yes
30% vs. 20%	0.0418	3	2.207	0.31	No
20% vs. 10%	0.0564	3	2.98	0.143	No

A.12. The ratio of vimentin-positive cells (Day 70)

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	2	0	0.371	0.0215	0.0152
Sample-20%	2	0	0.52	0.0176	0.0124
Sample-30%	2	0	0.591	0.00644	0.00455

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0.0502	0.0251	92.707	0.002
Residual	3	0.000812	0.000271		
Total	5	0.051			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
30% vs. 10%	0.219	3	18.862	0.002	Yes
30% vs. 20%	0.0706	3	6.071	0.047	Yes
20% vs. 10%	0.149	3	12.791	0.006	Yes

A.13. The ratio of SMα-actin-positive cells (Day 70)

Group Name	N	Missing	Mean	Std Dev	SEM
Sample-10%	2	0	0.196	0.000027	1.91E-05
Sample-20%	2	0	0.257	0.0118	0.00832
Sample-30%	2	0	0.235	0.00325	0.0023

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0.00385	0.00193	38.776	0.007
Residual	3	0.000149	4.97E-05		
Total	5	0.004			

All Pairwise Multiple Comparison Procedures (Tukey Test):
Comparisons for factor:

Comparison	Diff of Me	p	q	P	P<0.050
20% vs. 10%	0.0613	3	12.309	0.007	Yes
20% vs. 30%	0.0225	3	4.514	0.098	No
30% vs. 10%	0.0388	3	7.795	0.024	Yes

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