Chapter 4

INTERPENETRATING POLYMER AND TISSUE NETWORKS

This work has been done in collaboration with graduate student Joyce Huynh during her first year, who assisted with *in vivo* treatment of rabbit eyes. Undergraduates Dennis Ko and Meredith Wiseman contributed to the study of fluorescent molecules within the sclera.

4.1 Tissue Engineering

Tissue engineering is an attractive approach for treating keratoconus and degenerative myopia because of the potential to replace or reinforce old, diseased, or malfunctioning tissue. Hydrogel based materials are traditionally used in tissue engineering because of their adjustable mechanical and chemical properties, biocompatibility, and biodegradability.[1] These characteristics have been employed in biosensor coatings, drug delivery devices, the encapsulation of cells, cell delivery to tissue, and the creation of biodegradable scaffolding for new tissue growth.[2-4] Photopolymerizable hydrogels can be injected as a liquid and then solidified by exposure to light in a controlled, localized manner.[5] This method has demonstrated usefulness in filling voids in bone,[5-7] in wound

healing, and in creation of an engineered tissue, e.g., cartilage in a specified location and shape.[4, 6-11]

Poly(ethylene glycol) (PEG) or PEG-based materials have been widely used in hydrogel and tissue engineering applications, and PEG-diacrylate has been approved by the FDA for use in the body. PEG-based hydrogels formed by photopolymerization in the body have already shown clinical applicability in treatment of lung and dural tissues.[12, 13] PEG-based macromers may have varying-length PEG chains, which alters the solubility of the macromers and, upon polymerization, can create different crosslink densities (or modulus) of the polymerized network. By incorporating appropriate amino acid sequences along the PEG chains, the matrix can contain cell-binding domains, enzymatically degradable sections, growth factors, etc. This allows for artificial direction of cell growth and behavior. Also, PEG can be functionalized with varying end groups such as methacrylate, to give different reactivities. Radical generation by the photoinitiator initiates reaction of the endgroups, which form bonds with other macromers and/or with the tissue. Because of the customizability of PEG-based macromers, they have been chosen for use in our preliminary treatment mixtures.

While literature on tissue engineering typically focuses on the filling of voids, surface modifications, and temporary support for growing tissue, an alternative approach that we have studied for the cornea and sclera is the creation of an integrated polymer network within the tissue. By first allowing the tissue to imbibe photopolymerizable material, crosslinking incorporates the new material inside the tissue. This interpenetrating network could increase structural support, and through covalent crosslinks, could lock pre-existing

structures in place and prevent tissue reconfiguration. In the present case, it might prevent excessive deformation or tissue remodeling in keratoconus and degenerative myopia. In addition, we have demonstrated that it can be used to anchor labels or other functional moieties into the tissue (illustrated in Chapter 3 using Fluorescein-PEG-methacrylate). In studies of the mechanical properties related to polymerization or grafting of PEG-dimethacrylate (PEGDM) into ocular tissues, we have examined 1) the concentration dependence of modulus increases in sclera, 2) the relationship of oxygen to the modulus increase in sclera, and 3) the stabilization of eye shape with and without an interpenetrating network.

4.2 Concentration & Oxygen Dependence of Increases in Scleral Modulus

The density of the new network (artificial and natural), or the degree of crosslinking is expected to determine the treated tissue modulus. For example, a controlled increase in tissue modulus might be achieved by increasing the concentration of PEGDM, which is expected to increase crosslink formation.

In addition to control of the final modulus, in the case of degenerative myopia, we desire selective polymerization within sclera and not within vessel walls that penetrate the posterior sclera (Figure 4.1a). Oxygen inhibition of radical polymerization is commonly observed,[14, 15] which could be an advantage in this situation. Because sclera is largely avascular, we can exploit the differential concentration of oxygen within vessel walls compared to adjacent sclera to control polymerization. In and around blood vessels where

oxygen tension is higher, polymerization will be inhibited; further away, in avascular portions of the sclera where the oxygen tension is lower, polymerization will occur.

To test these hypotheses regarding the effects of concentration and oxygen on photo-activated incorporation of PEGDM, we use oscillatory shear measurements of the mechanical properties of porcine sclera specimens before and after treatment.

Methods: Eyes from 3–4 month old swine were obtained from Sierra for Medical Science. Tissue was shipped on ice, in saline to maintain proper hydration and freshness. The fresh eyes (< 72 hours post mortem) were dissected to remove orbital fat and muscles, and then cut at the equator to separate the anterior and posterior globe. The episclera, retina, and choroid were removed before cutting a circular disk from the posterior pole with an 8-mm-diameter trephine punch. The center of the circular section was located ~10 mm from the optic nerve on the temporal side. The sections were immediately put on the rheometer for oscillatory shear tests at a frequency of 1 rad/sec and a stress amplitude of 5 Pa.

After initial modulus measurements on the rheometer, tissue sections were soaked for 1 hour in a solution containing a specific concentration (0, 1, 5, 10, or 25 % w/w) of PEGDM (550 Dalton) with 1% 4-benzoylbenzyl trimethylammonium bromide (Initiator A: Figure 3.1a) in Dulbecco's phosphate-buffered saline (DPBS). After soaking for 1 hour, excess solution was removed from the tissue surface by dabbing with a kimwipe. The samples were mounted in a humid chamber that allowed control of oxygen in the atmosphere (Figure 4.1b). The atmosphere was either argon or air during irradiation. After 5 minutes of equilibration in the chamber, a Thermo Oriel 500 Watt mercury xenon arc lamp was

used to irradiate with approximately 4 mW/cm^2 365 nm UV light for 30 minutes. Following irradiation, the samples were rinsed for 4 hours in DPBS to remove unreacted macromer and initiator, and then modulus was measured again.

The storage modulus of each specimen was measured prior to the treatment protocol and again afterward. The same frequency and stress amplitude (1 rad/s, 5 Pa) were used for all measurements. The frequency and stress amplitude were chosen from the range of values where the modulus was in the linear regime. The temperature used for the soak and atmospheric chamber during irradiation was room temperature, and rheological measurements were done at 37ºC. For each specimen, the "% change in G′" was evaluated as $100x(G'_{after}-G'_{before})/G'_{before}$. Four specimens were examined for each condition: the mean and standard deviation are shown by the symbols and vertical bars in Figure 4.1c.

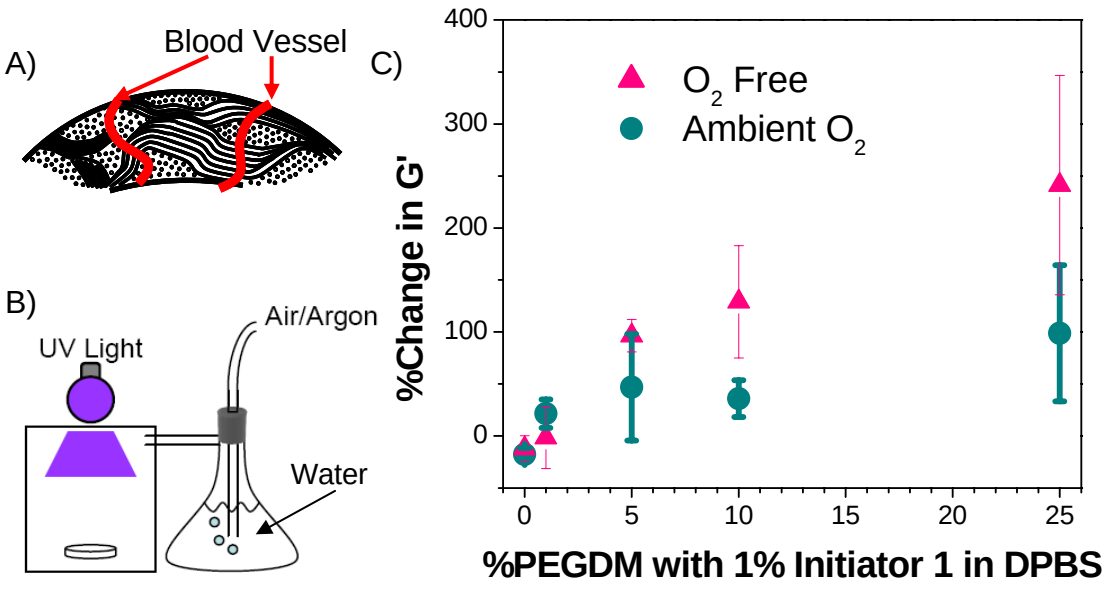

Figure 4.1. A) Schematic illustration of the collagen network of the sclera, depicting oxygen-rich blood vessels that should be avoided during polymerization. B) Humid chamber to provide UV light to tissue samples while maintaining proper hydration in different purge gases. C) Change in storage modulus of porcine sclera tissue sections at a frequency of 1 rad/sec, and oscillatory shear stress amplitude of 5Pa as a function of the concentration of PEGDM (0–25% w/w in DPBS) used in the treatment solution (see text). Samples received UV irradiation in either ambient O_2 (air) or no O_2 (argon). Results indicate a concentration-dependent increase in modulus. Presence of oxygen clearly decreases effectiveness of the reaction. Each data point is an average of 4 samples.

Results: A statistically significant increase in modulus was observed with as little as 5% PEGDM added to the solution. Increasing PEGDM concentration in the mixture generally increases the change in modulus of the sclera (Figure 4.1c). The change in tissue modulus for irradiation in the presence of oxygen is significantly less than that for irradiation under argon. At PEGDM concentrations of 25% w/w, the change in modulus while under argon was 240 ± 110 %, while in the presence of oxygen from air the modulus was 99 ± 65%. The modulus change in the presence of oxygen is less than half that achieved under argon for all concentrations of PEGDM 5% and above.

Discussion: The dose-dependent response of the tissue (increase in modulus with increasing %PEGDM) should enable the adjustment of individual treatments to achieve different desired end properties. The changes that occurred for the ~ 2 hour treatment with 25% w/w PEGDM under argon (240 ± 110 %) were comparable to those for a 24 hour 2% glyceraldehyde crosslinking treatment described in Chapter 2 (330 ± 110 %, Figure 2.16). Such shortening of treatment time would be beneficial for clinical application.

The inability to crosslink efficiently in the presence of oxygen could be a beneficial for this system. For the treatment of degenerative myopia it will be necessary to deliver drug around the back of the eye and close to the retina. The ability of oxygen to inhibit the reaction could provide a method by which strengthening of tissue is done everywhere except in close proximity to oxygen-rich vessels. This is especially relevant to prevention of crosslinking the short posterior ciliary arteries that penetrate sclera in the macular region and around the optic nerve. If drug and light did reach the choroid, the oxygen-rich layer could prevent damaging crosslinking there as well.

4.3 Crosslinking Without PEGDM

In addition to controlling tissue modulus by altering the PEGDM concentration, adjusting the treatment protocols (initiator concentration and soak time) and irradiation parameters (wavelength, intensity, exposure time) can be used to control the reaction. Shorter soak times prevent the drug from penetrating far into the tissue, and the light exposure controls the degree of activation. By examining these parameters, we discovered strengthening of the tissue was possible without any macromer, as illustrated by the following experiments.

Methods: Eyes from 3–4 month old swine were obtained from Sierra for Medical Science. Tissue was shipped on ice, in saline to maintain proper hydration and freshness. The fresh eyes (< 72 hours post mortem) were dissected to remove orbital fat and muscles. The whole eyes were soaked for 60 minutes in one of three solutions prepared in DPBS: a control of only DPBS, 0.3% I2959 in DPBS, or 20% PEGDM (550 MW) with 0.3% I2959 (I2959, Figure 3.1b). Afterward, sections of the eyes were irradiated with UV (~50 mW/cm^2, 315–405 nm, for 5 minutes) using a mask and then 8 mm disks were cut from irradiated and non-irradiated sections of the sclera. The samples were soaked for 24 hours in DPBS and then measured for modulus using oscillatory shear measurements. The difference in modulus between the exposed and unexposed regions was compared.

Results: Specimens from control eyes soaked in DPBS showed little difference in modulus between exposed and unexposed regions (14 ± 33%). The tissue sections from eyes receiving only initiator, or a combination of initiator and PEGDM both displayed increases in modulus in the exposed regions (290 ± 180%, and 150 ± 110% respectively). Due to the large scatter, there is not a significant difference between the treatment with and without PEGDM ($p > 0.05$ as determined using an unpaired t-test) so it is not possible to conclusively determine which treatment was better.

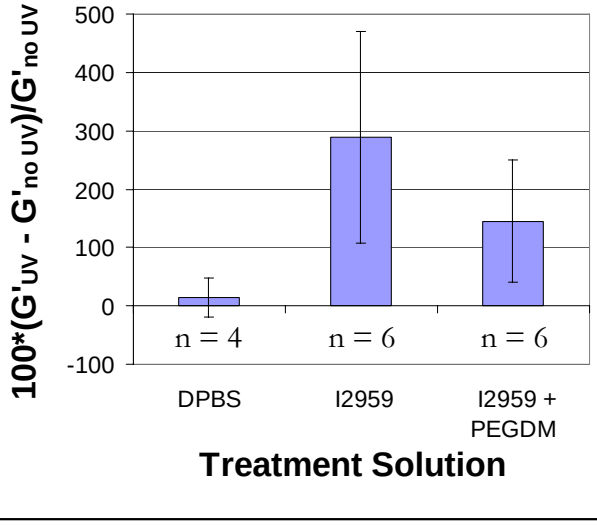

Figure 4.2. Eyes soaked in treatment solutions of DPBS, 0.3% I2959, and a mixture of 0.3% I2959 with 20% PEGDM were partially exposed to UV irradiation. Modulus values from tissue sections of the exposed region, G'_{UV} are compared to those of tissue sections from the unexposed region $G'_{no\ UV}$ to give a % difference of modulus due to treatment. Treatment with I2959 and treatment with the PEGDM initiator mixture both cause increases in modulus.

Discussion: These results indicate that treatments using only initiator and light have the capability of strengthening tissue by inducing crosslinking within the native components of the tissue. While the use of PEGDM allows the incorporation of degradable polymer sections, growth factors, and the possibility to further control stiffness through altering molecular weight, the use of just an initiator would greatly simplify the treatment and reduce the number of variables that must be studied.

4.4 *In Vivo* Treatment Comparison With and Without PEGDM

As discussed in the previous section, it is possible to increase tissue modulus with or without PEGDM present. Although there are advantages to either approach, they appear to have similar effects on modulus and it is not clear which will provide the best treatment *in vivo*. A set of experiments was conducted on rabbits in order to determine the efficacy of *in vivo* treatment with or without PEGDM while using the Eosin Y (EY) and triethanolamine (TEOA) photoinitiator system (Figure 3.1c). This system was chosen based on issues of safety discussed in Chapter 3.

Methods: The detailed methods for *in vivo* treatment delivery and irradiation are given in Chapter 5. For these experiments, 6 rabbits (12 eyes) received a subconjunctival injection of one of the following three solutions (Table 4.1): 1) control solution (DPBS), 2) 0.3 mM Eosin Y and 90 mM Triethanolamine in DPBS (10x EY), or 3) 10x EY with 10% PEGDM, 550 MW (10x EY w/PEGDM). After allowing 5 minutes for drug to diffuse into the sclera, the eyes were irradiated for 5 minutes using ~525 ± 16 nm, ~2 mW/cm^2 light. The animals were sacrificed on the same day that treatment was performed. The eyes were enucleated and then tested using the intact globe expansion method (Chapter 2).

Group	# of eyes	EY concentration	TEOA concentration	PEGDM concentration
1) DPBS	4	None	None	None
2) 10x EY	4	0.3 mM	90 mM	None
3) 10x EY w/PEGDM	4	0.3 mM	90 mM	10 % w/w

Table 4.1 *In Vivo* Treatment Comparison with and without PEGDM

Results: Treatment with either 10x EY or 10x EY w/PEGDM reduces the expansion of the sclera as compared to controls (SP, ED, SL, Figure 4.3). Because drug was only applied to the subconjunctival sclera, the corneas were untreated and changes in CP, CD, and CL are similar in treated and control eyes. The reduction in expansion along CD is most likely due to treatment effects at the limbus which constrain expansion of sclera around the edge of the cornea. Treatment without PEGDM may be slightly more effective at preventing expansion of the eyes, with average values of SP, ED, and SL being lower than those from treatment with PEGDM.

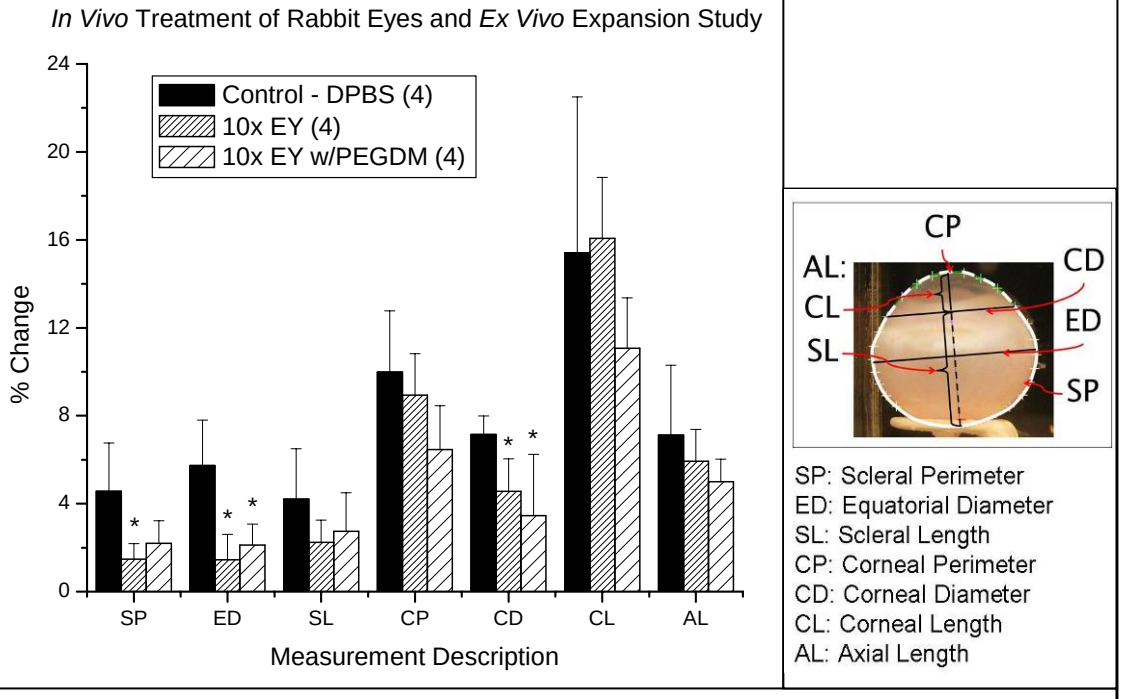

Figure 4.3. Rabbit eyes were treated *in vivo* and then examined using the intact globe method *ex vivo* in order to compare the treatment effects with and without macromer (PEGDM). Changes to scleral dimensions SP, ED, and SL are similar for both treatments, and there is some decrease in expansion along CD. Only the sclera was treated, so SP, ED, and SL are expected to differ from controls, while CP, CD, and CL may show minor changes. The measured ocular dimensions are illustrated in the side figure.

Summary: Previous work involving interpenetrating networks and tissue has demonstrated the use of these systems for ensuring hydrogel-to-tissue adhesion. West and coworkers have reported interfacial photopolymerization of PEGDM networks and tissue where the liquid form of the hydrogel penetrates into crevices in the tissue surface and upon polymerization, enhances adhesion between the final hydrogel coating and the tissue.[16, 17] In contrast to their work, we have demonstrated that interpenetrating networks can be used to increase the strength of tissue. The use of interpenetrating networks to control increases in the modulus of the cornea or sclera could be excellent treatments for keratoconus and degenerative myopia, particularly because of the potential to functionalize the network. Preliminary studies by undergraduate students Meredith Wiseman and Dennis Ko have shown the ability to deliver fluorescein-PEGM molecules within the sclera and immobilize them at a depth within the tissue (Figure 3.4), demonstrating functionalization and crosslinking of molecules inside the tissue.

Confocal microscopy of sclera from eyes soaked in the crosslinkable fluorescent molecule for 10 minutes reveals a gradient in fluorescence that varies with depth (Figure 4.4). The gradient in crosslinking is due to a concentration gradient from diffusion of molecules into the tissue, and due to light attenuation by the tissue. Future work that varies the crosslinking profile may illustrate differences in the mechanical properties (i.e., when PEGDM is present throughout the depth of the tissue instead of just the surface). Changing the soaking time, the time between soaking and irradiation, and the wavelength of light used for activation could all be used to modify the crosslinking.

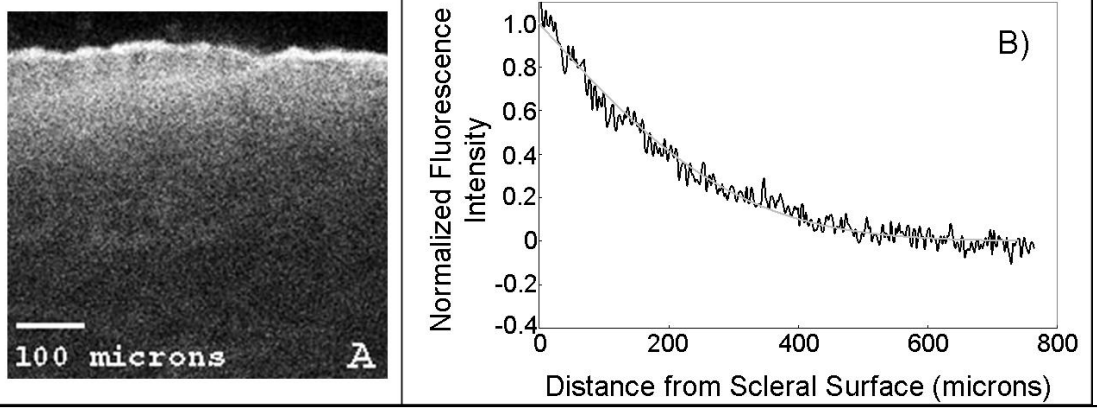

Figure 4.4. A) The confocal fluorescence image of porcine sclera shows fluorescence intensity of crosslinked fluorescein-PEGM within the tissue after a 10 minute soak, and B) a profile of the intensity as a function of depth demonstrates the gradient crosslinking within the tissue.

Results from this chapter indicate that the tissue can be strengthened by crosslinking of the native components of the tissue without the use of photopolymerizable molecules. Future studies could reveal certain conditions at which PEGDM plays the dominant role in strengthening the tissue, or other conditions at which crosslinking of the native tissue components dominates the strengthening. Studies on model systems using simple amino acids could reveal the interactions that are most important in the mechanism of crosslinking for both native tissue and interpenetrating networks.

The simplicity of using a photoinitiator-only system as the treatment is attractive because it can reduce the steps toward clinical use. In Chapters 5 and 6, we will focus discussion primarily on the use of EY/TEOA for treatment of degenerative myopia and keratoconus respectively.

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