Duquesne University

Annual Progress Report: 2006 Formula Grant

Reporting Period

July 1, 2009 – June 30, 2010

Formula Grant Overview

The Duquesne University received $71,320 in formula funds for the grant award period January 1, 2007 through December 31, 2010. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Modulation of Antitumor Immunity with Anti-Foxp3 siRNA Nanoparticles - The purpose of this project is to help develop alternative treatments for cancer patients. Conventional cancer drugs are highly toxic and often with poor efficacy. We propose to conduct research to attain a specific goal: to develop agents to remove a type of immune cells which are known to promote cancer.

Anticipated Duration of Project

1/1/2007 - 12/31/2010

Project Overview

The principal goal of this study is to develop novel agents to restore natural anticancer immunity by targeted eradication of regulatory T cells (Treg) cells. To this end, we propose to suppress the expression of the master transcriptional regulator in Treg cells, Foxp3. Since Foxp3 is a cytosolic protein, intracellular delivery systems have to be devised and developed.

An RNA interference strategy will be used to impede the recruitment of Treg cells in tumors. The hypothesis that shall be tested is that a localized interference of Foxp3 transcripts can downregulate Treg cells and upregulate anticancer T cell responses *in vivo*. Short interference RNA, or siRNA, targets messenger RNA with high specificity. In the cytosol, the antisense strand of the siRNA forms a duplex with the target mRNA and activates an enzymatic machinery to degrade the message in a specific manner.

Biodegradable nanoparticles will be used to deliver the siRNA in established tumors and in draining lymph nodes. Two specific aims are proposed:

1) To test the extent to which natural anti-melanoma T cell responses in vivo can be enhanced with NP-bound anti-Foxp3 siRNA. Tumors will be established in C57BL/6 mice by subcutaneous inoculation of B16-F0 cells.

2) To develop a second generation of nanoparticles with improved stability and RNA loading. A cationic polymer will be covalently attached to poly-(d, l-lactide-glycolide) particles. This vector will be tested for uptake in cultured mouse splenic T cells.

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Expected Research Outcomes and Benefits

As an outcome of the research proposed, we expect to determine the effects of using anti-Foxp3 siRNA nanoparticles to inhibit tumor growth *in vivo*. We will delineate the mechanisms of the response by measuring the balance of cytolytic (interferon-gamma-secreting) and regulatory (Foxp3-expressing) T cells in the animals. Guided by precise and sensitive spectroscopic methods (infrared and mass spec), we will optimize methods of chemical modification of polymeric paricles aimed at improving RNA loading and delivery. The studies have broad implications in the development of cancer vaccines and immunotherapy.

Summary of Research Completed

In this reporting period, we have investigated a fundamental issue in the delivery of siRNA into cells. A limiting factor in intracellular delivery of biologics is sequestration by endosomes. As part of specific aim 2, the goal is to understand and ultimately manipulate endosome burst in order to enhance RNA delivery. Our group has postulated that the cationic peptide, O10H6, can trigger endosome escape, due to its buffering capacity at pH 5-7. Results presented here center on the transport behavior coupled with the elastic membrane (modeling endosome) response which allows for the model to examine how an endosome expands due to increasing osmotic pressure, and allows for study and development of burst applications.

Background: Exogenous materials, including nucleic acids complexed with polycations used in this project, enter T cells through the process of endocytosis. In this process the cell extends and engulfs the surroundings, including a vaccine when present, for intake; the RNA is then enclosed in an inclusion known as an endosome. With the foreign species (RNA) encased, this endosome is then transported to the interior of the cell. While the endosome provides a mode of transport for vaccine uptake, it also acts to destroy and break down its contents via fusion with enzyme-rich lysosomes. It has been postulated that inducing endosome burst (lysis) may be an effective

way to affect the release of endocytosed RNA prior to the onset of degradation.

While there are a large number of biological transport protein types, they fall into three general classes: pumps, cotransporters, and channels (Figure 1). Each of these proteins transports fluid and charge across a membrane through various methods. In biological systems the pumps often provide the initial impulse for active response. Pumps break down the biochemical fuel ATP and use the resulting energy to transport an ionic species across the inclusion membrane against its electrochemical gradient. This action establishes a gradient which serves as the driving force for the cotransporters, exchangers, and channels. Cotransporters move a species along its electrochemical gradient and use the resulting energy to move a secondary species against its gradient. Exchangers similarly use the downhill motion of one transport process to drive the uphill transport of another, but unlike cotransporters they transport the species in opposite directions. Finally, channels serve as a passive transport pathway with voltage gating. Channels allow for species to transport out of or into the inclusion, but require the membrane to be at a certain potential before they open.

Method: A model that studies the final pH equilibrium of an endosome (with no proton sponge) has previously been created by Rybak et al. These authors note that several transporters traditionally exist in the endosome membrane: H+ ATPase, Na+ K+ ATPase, and Cl- Channels. Grabe and Oster have also studied the acidification of endosomes. Their studies indicate that the endosome membrane is also permeable to Cl- K+ diffusion and that Na+ K+ channels are also present. Freeman et al have recently presented a simulation of the early endosome. The primary active components considered include flow across Na+/K+ ATPase (pumps), H+ ATPase (pumps), Na+ channels, and K+ channels, as well as H+, Cl-, and water diffusion. In addition the membrane properties including bilayer membrane elasticity and rupture were considered. Concentrations of each of the species present (water, Na+, K+, H+, Cl-) were tracked in the simulation resulting in predictions of the evolution of internal pH, internal pressure, and volume change. The model was calibrated with experimental parameters available in the literature and subsequently similarly validated. The validated methodology of Freeman et al is applied in this study to test the hypothesis that introduction of a proton sponge may affect early burst.

To introduce proton sponge behavior to the system of simultaneous equations, several equations are introduced to model the proton accumulation and the impact of multiple sponges with different pKas. First, the sponge concentrations are influenced by the Henderson-Hasselbalch equations.

$$\langle x \rangle = \frac{10^{pKa-pH}}{1+10^{pKa-pH}} \quad (12)$$

< X > is the chance of protonation for a selected free proton, pKa is a measure of the strength of the selected sponge, and pH is the measure of the interior pH. When the pKa is equal to the pH, the chance of protonation will be 50%, or half of the transported protons will bind to the sponge. However equation 12 only accounts for a single protonation site. Onufriev, Case, and Ullman [Biochemistry,Vol 40, 2001, pg 3413-3419] created a model for multiple interacting sites which is implemented in the model. The drawback of this system is that it is not truly rate dependent and therefore volume expansion cannot be directly simulated. The equation may not

be modified to yield a rate reaction between free protons and attached protons. However, since the relationship between the number of free protons and attached protons has a small impact on the other terms present in the model, this is deemed acceptable. A second limitation is the equation assumes an infinite supply of protonation sites. This is remedied in the current model by introducing a decaying chance of protonation once the selected sponge approaches a theoretical limit. Naturally as the sponge accumulates more protons the availability of free locations will diminish, reducing the attachment rate. It is assumed that the chance of protonation is reduced in a linear fashion as the available positions are reduced.

$$f = \left(1 - \frac{[X]_{current}}{[X]_{max}}\right) \quad (13)$$

Multiple sponge equations are implemented using the methods from Onufriev et al. These sponges are allowed to interact through defining a set of constants a_{ij}.

$$\langle x_i \rangle = \sum_{j}^{N} a_{ij} \frac{10^{pKaj-pH}}{1 + 10^{pKaj-pH}} \quad (14)$$

The total chance of protonation for sponge i is determined by summing up the protonation chances for all neighboring sponges and multiplying them by an interaction coefficient a_{ij}. This allows the model to account for sponges competing for free protons and sharing available protons and other complex behavior.

This system of equations provides the framework for testing the proton sponge hypothesis. The equations are solved using fifth order runge kutta methods.

<u>Results</u>: The internal pH was plotted and compared (figure 2). Remember from previous studies that for the endosome case that proton diffusion plays a large role in determining the equilibrium pH. For the case with no sponges (and therefore minimal buffering), the pH rapidly reached this equilibrium pH. At this point the rate of proton diffusion becomes equal to the rate of transport from the proton pumps, and the pH stagnates. For the proton sponge case, we observe a buffering behavior. While the pH still decreases to a similar level, it does it at a much lower pace. Because the internal pH is held at a higher value, the proton diffusion is decreased, allowing for the proton pumps to continuously provide protons to the sponge for a longer period of time. This is one of the reasons for the increased osmotic pressure, the elevated membrane potential, and the eventual expansion of the endosome.

Several test cases were created with various combinations of sponges. For each case the interaction coefficients were assumed to be beneficial (sponges did not compete for protons). Four cases were created, one with 8.5 pKa sponges, one with 7.5 pKa sponges (same sponges seen in previous figures), one with 6.5 pKa sponges, and finally a case with all three of these sponges mixed together. The predicted volume responses with a comparison to the no-sponge case may be seen in figure 3.

Several behaviors may be observed. First, all of the cases considered are predicted to induce

burst. Figure 3 illustrates the point at which the elastic bilayer membrane reaches critical area strain. Thus the predictions are again supportive of the proton sponge hypothesis.

Presuming that the hypothesis is correct, the next goal is to use this understanding to control burst. It is therefore instructive to consider the variations in the prediction trends beyond the noted burst point, where it is assumed that these trends could be induced below the rupture point, for instance, via control of sponge quantity. From this perspective it is next noted that the stronger sponges initially expanded at a higher rate but reached a plateau and stopped expanding. This is because a sponge with a higher pKa will have a higher level of protonation at the beginning of the simulation. This will cause the sponge to reach its maximum capacity sooner (which, as noted above, is somewhat less than the other cases because sites are expected to become populated en route to the endosome). For this initial study the maximum capacity was assumed to be several moles, which allows the higher pKa sponges to still retain a significant amount of buffering capability even with the increased protonation. The cases with the 7.5 pKa and the 6.5 pKa sponges demonstrated constant expansion.

The mixed case resulted in a lower amount of expansion when compared to the pure cases. Initially the combination expanded rapidly due to the 8.5 pKa sponge, however the sponge reached its maximum capacity about 15 minutes into the simulation. This cessation of flow caused a reduction in the other sponges' uptake (assuming linked behavior), causing the net combination of sponges to underperform in comparison.

From the plots it appears that the 7.5 pKa sponge yields the best results. Increasing or decreasing the sponge pKa is counterproductive. With this knowledge, the study by Funhoff et al. (*Biomacromolecules*, 2004; 5: 32-39) may be revisited and confirmed. A sponge combination with given pKa values of 9.3 and 5.5 was created, and the resulting volume behavior was plotted which may be seen in figure 3.

This figure indicates that this combination of sponge pKas results in negligible expansion of the endosome – the peak volume reported is 0.0675 μm^3, while the peak volume reported for a zero-sponge case (see figure 2) is nearly identical at 0.0674 μm^3. The reason for this behavior is that the stronger pKa sponge is near-complete protonation at the onset of endocytosis. The weaker sponge is unable to absorb protons before the pH plateau behavior occurs. Both of these result in negligible expansion of the endosome, confirming that specific combinations of sponge pKa will not result in endosome burst. Thus, based on the transient analysis approach employed here the seeming contrarian result of Funhoff et al. in fact simply fails to select the correct sponge.

The proton sponge hypothesis is supported by the predictions of the system of equations selected for this study. The implication is that endosome burst may be achieved, and ultimately controlled, through the introduction of one or more proton sponges alongside RNA molecules. Per this study, the proton sponge caused the expected expansion of the endosome, along with the expected changes in internal concentrations and conditions.

<u>Conclusion</u>: The results indicate that sponge pKa is important when attempting to induce endosome burst. A sponge with a high pKa will be protonated prior to endosome entry, while a sponge with a somewhat lower pKa will be unable to yield significant expansion before the

proton diffusion term is equal to the proton pump flow, causing the pH plateau and limiting further expansion.

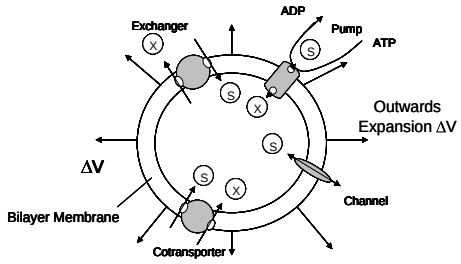

Figure 1 - Proteins Penetrating a Generalized Inclusion Enable Active and Passive Selective Transport of Species Through the Elastic Membrane

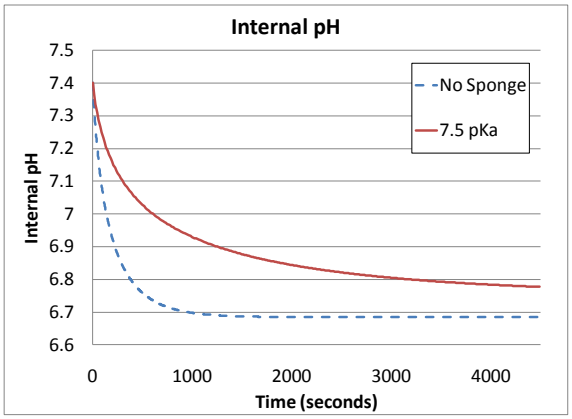

Figure 2 – Internal pH predictions

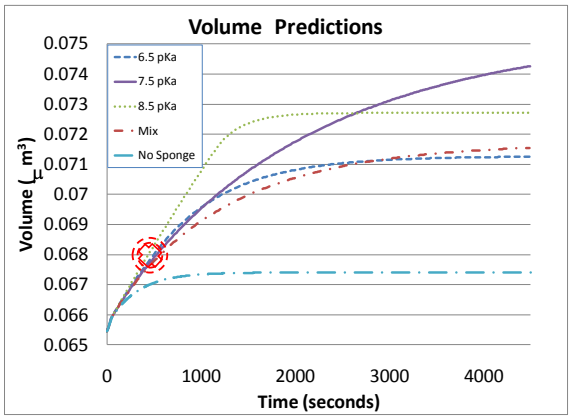

Figure 3 – Volume Predictions for varied sponges – the red marker indicates point of expected burst, all 4 cases meet the burst criteria

Research Project 2: Project Title and Purpose

Developing an Ophthalmic Estrogen Treatment for Cataract Prevention - The purpose of this study is to develop a topical estrogen therapy for application to the eye. Since estrogen has been shown to reduce the risk of cataract development, applying this estrogen therapy directly to the eye should provide the protection to the lens while limiting the amount of estrogen in other less desirable tissues, such as the breast. Using appropriate animal models, we will test whether this new ophthalmic estrogen therapy is safe to apply into the eye and supplies sufficient levels of estrogen to the lens. In addition, we will confirm that only minimal levels of estrogen enter the bloodstream to affect other tissues outside the eye.

Anticipated Duration of Project

1/1/2007 - 12/31/2010

Project Overview

The high risk of cataracts in women is believed due to the sharp decline in estrogen levels that occur after menopause. Exposure to estrogen has been determined in human and animal studies to be protective by reducing the risk of cataracts. Although the clinical and preclinical studies suggest estrogen therapy may reduce the risk of cataracts, many women are concerned about taking systemic estrogen treatments for fear of increasing other risks, especially breast cancer. In addition, the concept of giving systemic estrogen therapy would not be welcomed by the male population. Therefore, our goal is to develop pH-based in-situ gelling aqueous solutions containing 17β-estradiol locally to eye tissues with minimum availability to other tissues via the blood. Ophthalmic drug delivery has to overcome several challenges such as wastage of drug by rapid naso-lachrymal drainage, increased tear turnover upon instillation of the formulation and inherent physicochemical properties of the drug molecules (e.g., poor water solubility of a hormone like estrogen). Moreover, because the eye is a vital sensory organ in the body an additional challenge is to make the formulation compatible or safe with ocular tissues. Excipients or non-active ingredients such as carbopol and hydroxypropyl methylcellulose polymers that are already present in other commercial ophthalmic formulations such as Ultra Tears®, Isoto-Atropine (0.5%) and Tears Naturale® (Alcon Pharmaceuticals), Refresh® (Allergan) and Teargel® liquid gel (Adcock Ingram) will be used. An optimal gel system that will facilitate longer residence on the ocular tissue will be selected. Ocular irritation (watering eye, redness and swelling) studies will be performed on six male albino rabbits using a commercially available polymer base in order to establish less or no side topical or toxic effects. Lack of systemic side effects and local delivery will be established through pharmacokinetic (through absolute bioavailability) studies involving use of 12 rabbits from the same group designated for the irritation studies. Rabbits and humans have comparable naso-lachrymal duct morphology, therefore absorption of the topical ophthalmic formulations from the rabbit's eye will be predictive of that of humans. Investigation of known estrogen-dependent responses in the lenses of our transgenic mouse model (ERΔ3 mice) will verify that the drug is supplying sufficient estrogen to the desired target tissue with little to no effects on other non-ocular, estrogen-responsive tissues (uterus and mammary gland). If this therapy is efficacious and safe, it will pave the way for testing the potential of estrogen for cataract prevention.

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Expected Research Outcomes and Benefits

Cataracts are very common, with the highest risk in aging women. Currently, there are no therapies that can delay or prevent cataract development. However, studies on estrogen in women and animal models indicate it has promise to protect the lens from developing cataracts. In this study, we will develop and test a new drug for applying estrogen directly to the eye, which will be used in future studies for testing this hormone's potential to reduce cataract risk. Tests of the new estrogen formulations in the eye are expected to reveal very minimal or lack of irritation based on the fact that the chemicals used to make the estrogen gel (non-active ingredients) are already proven to be safe for application to the eye. In addition, the fact that the gel-based system being used will increase the time the drug stays in the eye and eyelids, thereby allowing the estrogen to exert its effects for a longer period. If minimal irritation is observed, it may be a result of the estrogen drug itself. However, this is not expected because there are some similar hormones that are already used for direct application to the eye. Moreover, a very low concentration of the estrogen will be used in the irritation studies, which should also eliminate or significantly reduce irritation. Similarly, for the studies measuring the amount of estrogen that enters the eye in proximity to the lens, the maximum concentration of the drug should be high enough to protect the eye from cataracts, as will be proven in the mouse studies. In contrast, the amount of estrogen that enters the blood after application to the eye should be significantly low to minimize any effects on other estrogen tissues. Therefore, in the mouse studies, there should be little evidence of estrogen action in the uterus and mammary gland in contrast to the stimulation of estrogen responses in the eye (lens). The development of this new ophthalmic estrogen therapy will allow future testing on the benefits of estrogen in the eye, such as delaying cataract development. As delaying cataract development by one decade could reduce the number of cataract surgeries by almost half, this drug could provide a significant health benefit to the elderly.

Summary of Research Completed

<u>1). Development of an optimized in-situ gel forming ocular estrogen formulation:</u>
In-situ gel forming ocular 17β-estradiol (E_2) formulation was prepared according to the method described in previous reports. The optimized formulation was chosen based on the design of experiments approach in which two levels of polymer (0.1 & 0.3%w/v) and three drug loads (0.025, 0.01, & 0.001%w/v) were varied to study their effect on *in-vitro* performance characteristics of the in-situ gel ocular formulation. All six formulations exhibited desirable

characteristics of an ocular formulation i.e. clarity>85%, pH in the range of 6-7, drug assay of 90-110%, osmolality in the range of 265-325 mOsm/kg, and anti-microbial efficacy. Drug release from these *in-situ* gel formulations was sustained as 60-100% of the drug was released in 8-10 hours. The respective cumulative drug release profiles are shown in Figure(s) 1(a) & (b). Multivariate analyses of the drug release metrics (i.e. $t_{80\%}$ and release rate constant (K)) indicated the influence of polymer level and drug loading to be significant ($p<0.05$). All formulations exhibited shear thinning behavior as shown in Figures 2 (a)& (b), and increase in polymer level or drug load resulted in higher viscosities. Mixing the formulation with the STF resulted in i*n-situ* phase change to gel state, as elastic modulus (G') in all cases as shown in Table 1 was greater than viscous modulus (G"), and phase angle (**δ)** was <45°. Further, higher polymer levels and lower drug loads resulted in stronger gel structures.

Based on these results formulation containing 0.3% polymer and drug load of 0.025%w/v was chosen as an optimized formulation. The stability testing of this optimized formulation is ongoing and the 2 month time point indicated good stability of the ophthalmic drops at the various temperatures tested. The stability testing will continue up to 6 months. Using this optimized formulation the animal testing for irritation, delivery to the eye and systemic bioavailability, and estrogen-induced responses in the lens are described below.

2) Draize Test to verify the E_2 formulation is non-irritating

The Draize test is the classic and required test for irritation needed for ophthalmic formulations prior to clinical testing. Therefore, the 17β-estradiol formulation was tested for irritation using the Draize test. The mid polymer concentration (0.3%) has better gelling characteristics than the low concentration (0.1%), so it was used for the Draize test. A 30 μl drop (0.3% polymer and 3.75 μg/eye E_2) was delivered to one eye twice daily for 3 days (d0, d1, d2) in 6 male rabbits. The eye receiving the formulation was randomized among the rabbits (3 received E_2 drops in the right eye and 3 were tested in the left). Rabbits were monitored for any signs of irritation at 6 hrs, 1, 2, 3, 6 &, 7 days post-administration and scored according to Draize scale. No signs of irritation or corneal changes were observed in any of the treated eyes. The only observations noted were a slight discharge in a minority of the rabbits. See Figure 3 for representative photographs, the control and treated eyes from one rabbit. The final Draize score was 0.67 indicating the formulation is non-irritating. Therefore, this formulation would be expected to be non-irritating in our future clinical trials.

3) Effective delivery of E_2 to the eye with low level systemic E_2 bioavailability

For the ophthalmic formulation to reach the lens, it must permeate the cornea and acquire detectable levels in the aqueous humor. Thus, to determine if E_2 permeates the cornea, if higher levels reach the aqueous humor compared to systemic circulation, and if levels remain higher for longer periods due to the in situ gel not being washed out of the eye, rabbits were either injected with 7.5 μg E_2 iv or given 30 μl drops with the equivalent dose/rabbit (3.75 μg/eye with both eyes treated, 0.3% polymer). Aqueous humor E_2 levels were only measured for the ophthalmic delivery. Blood and aqueous humor samples were collected at multiple time points between 15 min to 4 hr and E_2 levels were assessed by radioimmunoassay (RIA). For these assays, we chose a dose of E_2 which would enable us to measure the estradiol levels in the serum and aqueous humor by RIA and to observe the time of elimination.

For the aqueous humor kinetics, the AUC(0-t) was ~53 hr.ng/ml compared to the serum level where the AUC (0-t)was 219 hr.pg/ml indicates 1) that the level detected in serum was almost 250 times lower than the concentration in the aqueous humor and 2) that the drug was able to permeate the cornea membrane. Both the systemic and ophthalmic treatments result in a rapid spike in estradiol levels within 15 minutes after administration. Within 1 hour after systemic administration of E_2 (iv), its detectable serum levels drop to levels approaching the 0 timepoint and return to baseline levels by 4 hrs (see panel A in Figure 4). These data indicate E_2 in serum has a short half-life as has been previously reported (15-20 minutes). In contrast, levels drop more slowly in the aqueous humor and are still high (>100 pg/ml) compared to its undetectable baseline levels (see panel B in Figure 4). Serum levels in the rabbits treated with the ophthalmic drops were substantially lower compared to animals receiving the iv injection; plus, these levels also decline to baseline levels within the 4 hr test period. These data indicate that only low levels are systemically delivered compared to the high levels that are present in the aqueous humor. Due to the effective delivery of high doses to the aqueous humor, future studies using lower doses that do not result in detectable serum levels will likely be able to protect lens transparency.

To determine if an estrogen-induced response would be detectable due to systemic absorption at various estrogen doses after ophthalmic delivery, a classic uterotropic assay in ovariectomized (OVX) mice was used to determine if ophthalmic delivery of E_2 resulted in stimulation of the estrogen responsive uterus. After 3 daily treatments with E_2 by injection (sc) or ophthalmic drops, uterine weight (normalized to body weight) was measured on day 4. For the mouse study, 3 E_2 doses (0.4, 0.04, and 0.004 μg/mouse) with the low polymer concentration (0.1%) were tested for the ophthalmic drops vs. the same concentration by subcutaneous injection. All three doses of E_2 administered systemically resulted in uterine stimulation; whereas, only the two higher doses delivered to the eye increased uterine weight compared to vehicle-treated mice (Figure 5, panel A). However, for the 0.04 μg/mouse ophthalmic dose, the level of stimulation was reduced compared to the systemic delivery. In addition, the eye drops containing the lowest E_2 dose did not increase the uterine weight, unlike the same dose administered by sc injection.

Mice are highly sensitive to low levels of E_2 due to the lack of peripheral aromatization; therefore, the systemic levels that stimulate the uterus with the 0.04 μg/mouse eye drops may not result in a similar effect in women. However, in the mice, 0.004 μg E_2 did not cause uterine stimulation and it may be an effective dose in humans for cataract prevention (with adjustments for weight) based on the high levels of E_2 transferred to the aqueous humor in the rabbits.

The levels of E_2 that reach the systemic circulation may also be lower with the 0.3% polymer vs. the 0.1% used in the mice due to its better gelling properties. Therefore, we also compared two polymer concentrations (0.1% vs. 0.3%) at one E_2 dose (0.4 μg/rat) in the uterotropic assay compared to systemic administration in OVX Sprague-Dawley rats. Neither polymer concentration resulted in uterine stimulation in comparison to vehicle treated rats, unlike systemic delivery (Figure 5, panel B). These data suggest that the systemic levels of E_2 that occur after ophthalmic delivery with either polymer concentration are insufficient to stimulate the highly responsive uterus in the rat, but are in the mice. However, since the same E_2 dose was delivered to both species (0.4 μg/animal), the rats would have a lower effective dose (approximately 5-fold) if calculated based on animal weight (μg/kg). Even with this adjustment,

the rats do not show the uterine stimulation at the 0.04 μg/mouse dose, suggesting the rats are less sensitive to similar systemic estrogen concentrations or the bioavailability differs between the species. Since E_2 in both polymer concentrations did not stimulate the uterus, we cannot determine if the systemic bioavailability differed due to the polymer concentration.

4) In situ-gel formulation results in estrogen action in lens

The rabbit data indicates E_2 permeates the cornea, but to confirm E_2 reaches the lens to induce estrogen responses, we examined cataract induction and lenticular gene expression in the ERΔ3 mice. ERΔ3 mice are a transgenic mouse model which expresses a dominant negative estrogen receptor that results in cataract formation in the presence of high doses of estradiol (endogenous levels in intact females and exogenously administered estrogen in males, OVX females, and immature mice). Therefore, we used this model to test the ophthalmic formulations vs. systemic estrogen treatment in immature ERΔ3 mice for their ability to induce cataracts.

Grossly detectable cataracts were first observed by 3 wks after treatment, but none were observed in mice treated with vehicle (sc or drops). Cataracts were induced by all 3 E_2 doses of 1, 0.4, and 0.04 μg/mouse by ophthalmic or sc delivery. (The ophthalmic dose was determined based on delivery to both eyes, with each eye receiving half the injected E_2 dose.) At 0.4 μg E_2/mouse, cataracts were evident in both eyes by 6 wks post-treatment in all 7 animals with the 0.3% polymer formulation, with 6 of the mice having strong cataracts (as shown in Figure 6). However, none of the 8 mice treated with this E_2 dose/0.1 % polymer had visible cataracts in both eyes at the same timepoint. For systemic 0.4 μg E_2/mouse administration, none had strong cataracts, but 3 of 7 had opacities in both eyes by 6 wks post-treatment. These data demonstrate that ophthalmic delivery of E_2 with the 0.3% polymer concentration results in an earlier cataract onset vs. 0.1% polymer drops or systemic treatment. The better efficacy with the higher polymer content is likely due to its better gelling behavior in the eye. These data suggest that the 0.3% polymer concentration could be the better choice for clinical testing.

In the ERΔ3 mice, RNA expression of the *pax6* gene is increased in the lens at 6 hours after high doses of E_2 are injected (20 μg/mouse, sc). Therefore, we examined whether the expression of *pax6* would also be upregulated by ophthalmic E_2. RNA expression (by real-time RT-PCR) was examined in lenses of mice at 6 hours after treatment with increasing doses of E_2. The ophthalmic 0.4 μg of E_2/mouse (drops) treatment resulted in a significant increase in *pax6* expression vs. control, unlike the same systemic dose (sc) (Figure 7). No changes in *pax6* expression were noted for the injected or ophthalmic doses of 0.04 or 0.004 μg/mouse compared to vehicle (data not shown, n=4). These results verify that the ophthalmic delivery of E_2 is able to induce a known, rapid estrogen response in the lens, unlike the same dose of systemic E_2. Collectively, these findings indicate that we developed an effective ophthalmic delivery system for future testing for cataract prevention in preclinical models and clinical trials.

Figure 1: Cumulative drug release profiles of formulations containing three different drug loads at two different polymer levels a) 0.1% polymer b) 0.3% polymer

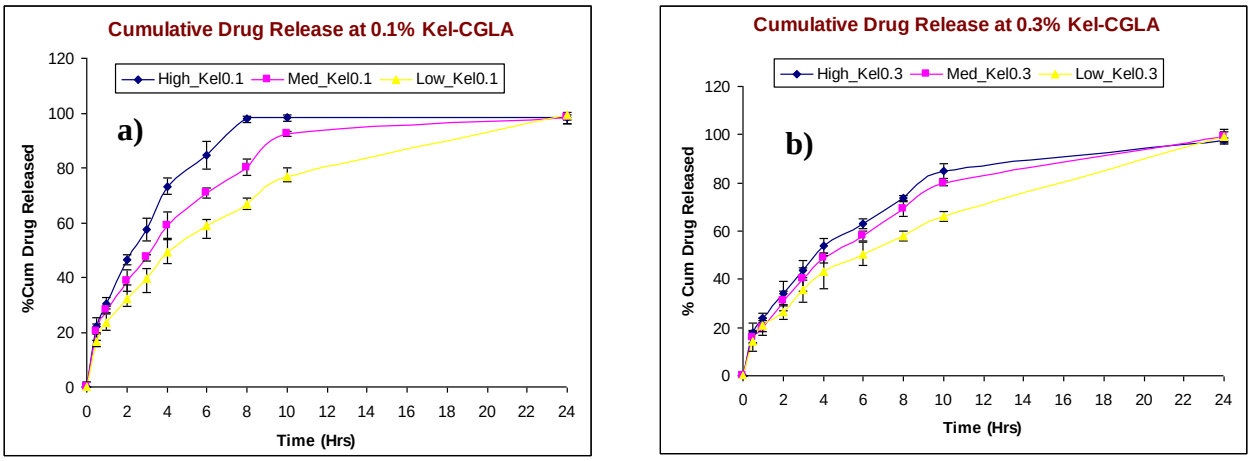


Figure 2: Viscosity vs. sear rate profile for formulations containing three different drug loads at two different polymer levels a) 0.1% polymer b) 0.3% polymer

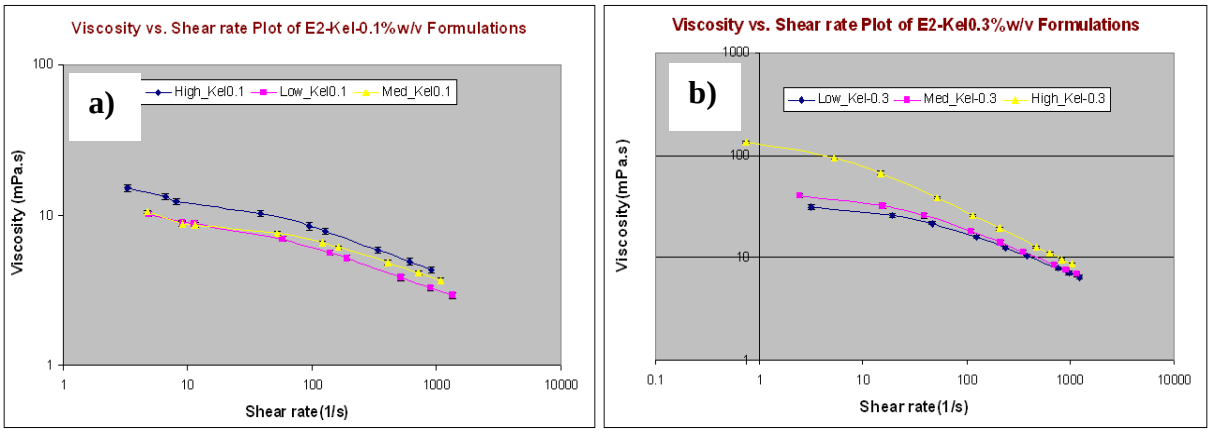


Table 1: Viscoelastic properties of the formulations measured in the presence of STF

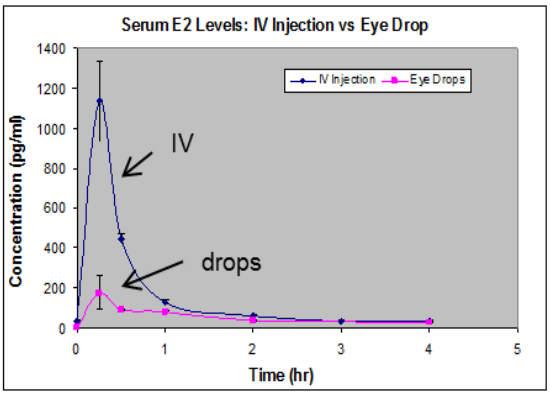
Viscoelastic Properties of E2 Formulations in the Presence of STF Measured in LVE at 5rad/s and 3% Strain					
Polymer Level	Kelcogel 0.3%w/v	Drug Load	G'(Pa)	G''(Pa)	Delta(°)
		High-0.025%w/v	0.56±0.09	0.44±0.06	38±1
		Med-0.01%w/v	0.56±0.04	0.40±0.11	36±2
		Low-0.001%w/v	2.9±0.11	1.02±0.09	19±2
	Kelcogel 0.1%w/v	Drug Load	G'(Pa)	G''(Pa)	Delta(°)
		High-0.025%w/v	0.13±0.02	0.08±0.004	32±1
		Med-0.01%w/v	0.05±0.006	0.03±0.001	31±1
		Low-0.001%w/v	0.41±0.04	0.12±0.02	16±1

Figure 3. E_2 Formulation in Draize Test in rabbit eyes Representative pictures are of a treated (E_2) and untreated eye in one rabbit. The picture on day 2 (d2) is after all 3 treatments in the rabbit and on day 7 (d7) is the final day of observation as outlined for the Draize test.

Untreated d2 Treated d2 Untreated d7 Treated d7

Figure 4. Serum and aqueous humor levels of E_2 at various times after ophthalmic delivery. Panel A represents the serum E_2 levels for the estradiol delivered systemically (IV) or topically to the eye (drops). Panel B denotes the E_2 levels in the aqueous humor after ophthalmic delivery. Note the different scales for the serum (panel A in pg/ml) being 1000-fold lower than the aqueous humor (panel B in ng/ml, black horizontal line is 1 ng/ml). The levels of E_2 in the aqueous remain elevated up to 4 hr after ophthalmic delivery, unlike in serum by either method of administration.

A.



B.

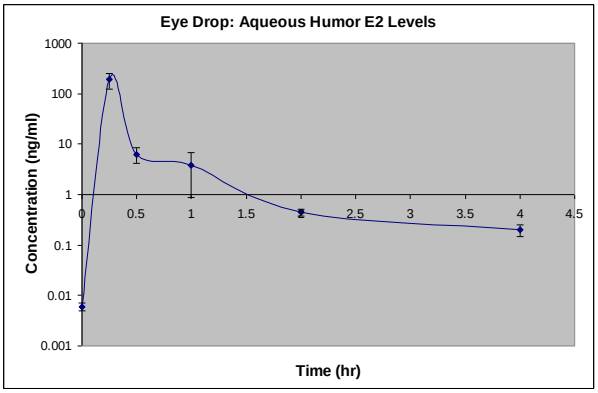
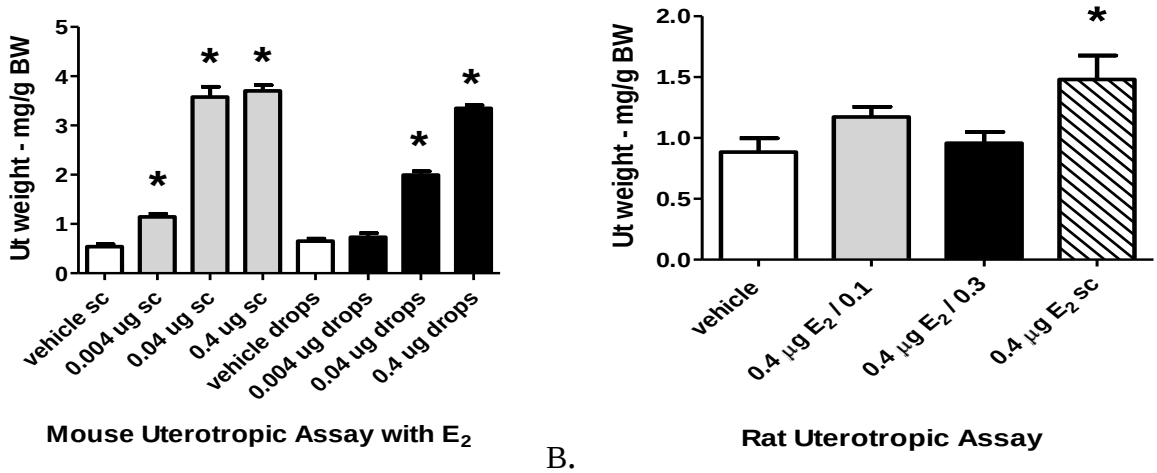
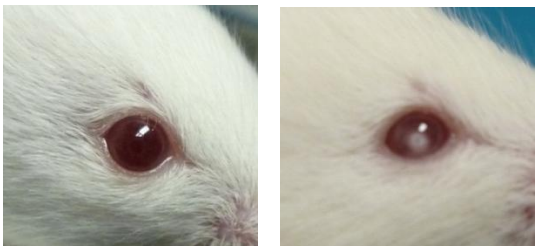


Figure 5. Uterine stimulation by E_2 after ophthalmic and systemic delivery in mice and rats. In the OVX mice (panel A), lower uterine weights are evident for the 0.04 ug drops vs. sc and no uterine stimulation is evident for the 0.004 μg drops group (1-way ANOVA $p<0.0001$, * indicates significance vs. vehicle by Tukey's test). In panel B: A uterotropic assay in immature Sprague Dawley rats compared the 0.1% and 0.3% polymer concentrations for the same E_2 dose (0.4 μg). Neither formulation containing E_2 resulted in significant stimulation of the uterus compared to the vehicle drop, unlike the injected 0.4 μg E_2 sc (1-way ANOVA, $p< 0.03$).



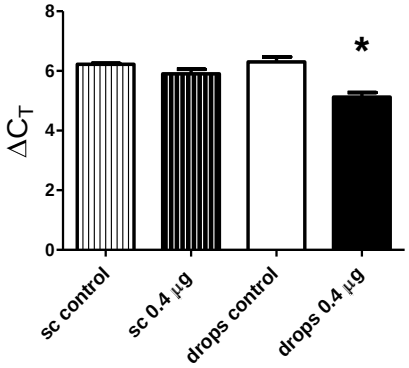
A. B.

Figure 6. Grossly detectable cataracts in ERΔ3 mice after ophthalmic E_2 administration. Daily treatments were given for 6 days in immature males and females (ovariectomized prior to puberty to prevent the endogenous estrogen's ability to induce cataracts). Photograph in the left panel shows an example of a strong cataract with E_2 drops compared to the vehicle drops (right panel) > 6 weeks after treatment.



vehicle drop E_2 drop

Figure 7. Expression of an estrogen-responsive gene in the ERΔ3 mouse lens.
Expression of pax6 was evaluated in RNA prepared from lenses 6 hr after treatment using real-time RT-PCR. Lower ΔC_T values reflect higher expression levels. The relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Expression of pax6 was increased 2.6 fold over the vehicle control. * indicates significance compared to all the other groups (1-way ANOVA, p=0.0003; it was significantly different than sc E_2 and both control groups by Tukey's test, n=4).



***pax6* Expression in ERΔ3 lenses**