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**“Subconjunctivally Implantable Hydrogels with Degradable
and Thermoresponsive Properties for Sustained Release of
Insulin to the Retina”**

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Abstract

The objective of this work is to develop subconjunctivally implantable, biodegradable hydrogels for sustained release of intact insulin to the retina to prevent and treat retinal neurovascular degeneration such as diabetic retinopathy. The hydrogels are synthesized by UV photopolymerization of N-isopropylacrylamide (NIPAAm) monomer and a dextran macromer containing multiple hydrolytically degradable oligolactate-(2-hydroxyethyl methacrylate) units (Dex-lactateHEMA) in 25:75 (v:v) ethanol:water mixture solvent. Insulin is loaded into the hydrogels during the synthesis process with loading efficiency up to 98%. The hydrogels can release biologically active insulin *in vitro* for at least one week and the release kinetics can be modulated by varying the ratio between NIPAAm and Dex-lactateHEMA and altering the physical size of the hydrogels. The hydrogels are not toxic to R28 retinal neuron cells in culture medium with 100% cell viability. The hydrogels can be implanted under the conjunctiva without causing adverse effects to the retina based on hematoxylin and eosin stain, immunostaining for microglial cell activation, and electroretinography. These subconjunctivally implantable hydrogels have potential for long-term periocular delivery of insulin or other drugs to treat diabetic retinopathy and other retinal diseases.

Introduction

Diabetic retinopathy (DR) is a chronic disease and one of the most frequent complications of diabetes, affecting 60-75% of persons with diabetes [1]. Retinal neuronal loss and increased vascular permeability occur in early stages of diabetes [2] and precede clinically evident vascular changes. Intensive systemic insulin therapy reduces retinal neural apoptosis in diabetic rats [2, 3], and intensive metabolic control with systemic insulin therapy reduces the risk of the development and progression of DR in diabetic human subjects (Diabetes Control and Complications Trial, 1994). Insulin rescues retinal neurons from cell death in a phosphatidylinositol 3-kinase dependent fashion [2] and intraocular injection of insulin restores the basal retinal insulin receptor activity in diabetic rats [3]. Taken together, these results suggest that insulin may have direct pro-survival actions in the retina. However, the degree of systemic insulin therapy is limited by the risk of hypoglycemia, including seizures and coma [4], and therefore, long term local delivery of insulin to the retina is needed.

Therapeutic drugs can be delivered to the retina via systemic or topical, subconjunctival, or intravitreal routes [5-8]. However, the task of adequately delivering drugs to the retina is challenging due to limited access to the retina and the blood ocular barriers. Oral or intravenous administration may deliver drugs to the retina but also carries the risk of systemic toxicity. Topical administration may be effective for the ocular anterior segment but is inefficient for retinal delivery due to the barriers of corneal epithelium, aqueous humor, blood-aqueous barrier and vitreous humor. Additionally, proteins and peptides may not be absorbed through the corneal epithelium. Intravitreal

administration is an effective means of delivering therapeutic levels of drug quickly with minimal systemic side effects, but it is an invasive procedure with complications such as cataract formation, vitreous hemorrhage, and endophthalmitis [9]. Subconjunctival administration has potential to be a minimally invasive method with the combined merits of both the topical and intravitreal administrations for effective sustained drug delivery to the retina [10-12].

Hydrogels have been used for controlled release of a variety of therapeutic agents including proteins and peptides during the past two decades [13]. A hydrogel implant offers advantages of good biocompatibility and mimics biological tissues [14]. Organic solvents can denature protein drugs, and thus it is desirable to load proteins into delivery systems including hydrogels in aqueous environment. There are a few hydrogel systems reported in the literature that achieve aqueous loading of insulin and release insulin for a few weeks. These systems are either sol-gel type of physical gels [15] that take a long time to form, which may cause fast burst release and result in tissue irritation and systemic toxicity; or chemically crosslinked gels with surfactants [16] that are very difficult to remove completely and also cause toxicity. In addition, the insulin loading efficiency in these reported hydrogels are not clear and the gel sizes are rather large (2.5 mL in ref. [15] and 157 μ L different units here in ref. [16]). In our previous work, we have developed thermo-responsive and biodegradable chemically crosslinked poly(N-isopropyl acrylamide-co-dextran-lactate-2-hydroxyethyl methacrylate) [also referred to as P(NIPAAm-co-Dex-lactateHEMA)] hydrogels [17] that can load bovine serum albumin (BSA) in PBS (pH 7.4) during the synthesis process and sustain BSA release for two weeks [17].

In this study, we modulate the components of the P(NIPAAm-co-Dex-lactateHEMA)] hydrogels and fabricate small hydrogels (2 mm diameter, 1.6 mm thickness, 5 μ L volume) for the purpose of implanting the hydrogels in the subconjunctival space of rat eyes to continuously release insulin to the retina. This approach has the potential to augment effects of systemic insulin and ensure adequate concentrations of insulin within the retina, without risking hypoglycemia for the treatment of diabetic retinopathy. We have studied the effects of the hydrogel composition and dimension on the release kinetics of insulin from the hydrogels; the stability and biological activity of the released insulin; and the *in vitro* toxicity and *in vivo* biosafety of the hydrogels after subconjunctival implantation in rat eyes.

1. Materials and methods

1.1. Materials

Dextran ($M_w = 15,000\text{-}20,000 \text{ g}\cdot\text{mol}^{-1}$) was purchased from Polysciences, Inc. Warrington, PA. The following materials were obtained from Sigma-Aldrich, Inc., St. Louis, MO: NIPAAm, 2-hydroxyl methacrylate (HEMA), 4-(N,N-diethylamino) pyridine (DMAP), N,N'-carbonyl-diimidazole (CDI), L-lactide, stannous octoate (SnOct_2), tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), fluorescein isothiocyanate (FITC), and bovine insulin (28 IU/mg). Bovine serum albumin (BSA, $M_w = 67,000 \text{ g}\cdot\text{mol}^{-1}$) was obtained from Fisher Scientific, Hanover Park, IL. All the chemicals were used as received. Deionized distilled water was used in all the experiments.

1.2. Fluorescent labeling of Insulin

Bovine insulin was dissolved in distilled water (5 mg/mL) at pH 3 and then sodium carbonate was added to adjust the pH to 9. Solution of FITC in DMSO (1mg/mL) was added drop-wise to the insulin solution and then the reaction was carried out at 4 °C overnight. The resulting solution was centrifuged at 12000 rpm using ultracentrifuge tubes with 2 kDa molecular weight cut off (Viva Science). The ultracentrifuge tubes allowed filtration of the un-conjugated FITC but retention of the FITC-conjugated insulin inside the tubes. The inside FITC-insulin-containing concentrated solution was re-suspended in distilled water and centrifuged again, and the process was repeated twice. Florescence of the filtrates was measured on a SPECTRAmax GEMINI EM fluorescence plate reader (Molecular Devices Corporation, CA). There was no significant amount of unconjugated FITC (< 0.007% of total FITC-insulin) detected in the last filtrate implying that the concentrated FITC-insulin solution was free of unconjugated FITC. The concentrated FITC-insulin solution was recovered from the tubes and lyophilized.

1.3. Synthesis of degradable Poly(NIPAAm-co-Dex-lactateHEMA) hydrogels

Dex-lactateHEMA macromer was synthesized using the procedure described previously [17], and its structure is shown in scheme 1. The lactide chain length (DP) of the lactate unit and number of lactide chains on dextran (DS) in the macromer were estimated by ¹H NMR. The DP affects hydrolytic degradation of the ester bond in the

lactide chain while the DS controls the degree of cross-linking (swelling) in the hydrogel.

Poly(NIPAAm-co-DEX-lactateHEMA) hydrogels were synthesized by UV-initiated free radical polymerization method. A prepolymer solution was prepared by dissolving NIPAAm/Dex-lactateHEMA at different weight ratios: 8/1, 6/3, and 4/5 (w/w), and 0.1 wt% Irgacure 2959 in a solvent containing 25/75 (V/V) ethanol/distilled water (as ethanol is biocompatible, we used 25 v% ethanol to increase the solubility of the Dex-lactateHEMA macromer). 2 μ L of solvent was used for each milligram of the combined weight of NIPAAm and DEX-lactateHEMA macromer.

A Teflon mold with well defined wells (2 or 4.5 mm in diameter and 1.6 mm in height) was fabricated. The wells were filled completely with the prepolymer solution, covered with a glass cover and irradiated with UV light ($\sim 500 \text{ mW/cm}^2$, EXFO Lite UV source) for 3 min. The resulting hydrogels were taken out of the wells and washed with distilled water twice for 10 minutes each. The washed hydrogels were frozen with liquid nitrogen and dried in a freeze-dryer overnight. The hydrogels without insulin will be referred to as “blank hydrogels” in the subsequent sections.

FITC-insulin loaded hydrogels were made by dissolving 15 wt% FITC-insulin and 10 wt% BSA in the prepolymer solution. 10-20 μ L of 1N hydrochloric acid was added for complete solubilization of FITC-insulin. The rest of the synthesis process was the same as detailed above for the blank hydrogels. Insulin-loaded hydrogels were made in a similar fashion except for using bovine insulin instead of FITC-insulin.

1.4. In vitro insulin release from hydrogels

The FITC-Insulin loaded hydrogels were transferred into glass vials containing 1 mL PBS at pH 7.4. The glass vials were placed in a 37° C water bath with mild shaking. 50 µL aliquots were withdrawn at predetermined time intervals from the release medium with addition of an equal volume of PBS solution into the release medium. The aliquot samples were collected in a 96-well plate. Fluorescence intensity of the samples was measured on a SPECTRAmax GEMINI fluorescence plate reader and the amount of released FITC-Insulin was estimated using a calibration curve.

Empirical power law (equation 1) was used to analyze the release kinetics:

$$M_t/M_\infty = kt^n \quad (1)$$

where k and n are constants that are related to diffusion coefficient and transport mechanism, respectively. M_t and M_∞ are the mass fractions released at time t and infinity, respectively. A value of 0.5 for n corresponds to diffusion controlled release while values higher than 0.5 are regarded as anomalous diffusion. A zero order release is obtained if n equals to 1.

1.5. Cells and media

R28 retinal neuron cells were seeded in T25-flasks at a density of 8,000~12,000 cells·cm⁻², and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), vitamin, essential amino acids, and penicillin/streptomycin at 37 °C with 95% humidity and 5% CO₂. The medium was changed every other day. The cells were harvested with trypsin (0.05% trypsin with 0.4 mM EDTA) when they were 80% confluent on the fourth day.

1.6. In vitro cytotoxicity of hydrogels to R28 retinal cells

Cytotoxicity of the hydrogels to R28 cells was examined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. R28 cells were seeded at a density of 50,000 cells·cm⁻² in a 48-well plate containing 500 µL medium at 37 °C for 48 h. Blank hydrogels were then incubated with R28 cells for 24 h. Wells containing cells alone were used as control. 25 µL of MTT solution (5 mg MTT/mL DMEM) was added to each well and incubated for 4 h. The medium was removed completely from the wells and 500 µL of 20% sodium dodecyl solution in dimethyl formamide/water was added to each well and incubated at 37 °C overnight. The absorbance of the reduced form of the MTT was measured at 570 nm on a µQuant plate reader (Biotech Instruments, Inc.) with background subtraction. Cell viability was calculated by dividing the absorbance of wells containing the hydrogels by the absorbance of control wells. Four replicate wells were used for each sample and control. Cytotoxicity of the hydrogels was also tested by incubating the hydrogels at 37° C in the R28 culture 50 µL medium for one week and then transferring the solution to a R28-seeded 48 well plate to perform the above described MTT test.

1.7. Stability of released insulin

Insulin loaded hydrogels were incubated in 1 mL PBS at 37 °C. 10 µL sample solutions were removed after 1 and 7 days of incubation. Tris Glycine native sample

buffer was added to the samples and the resulting solutions were run on a 12% Bis-tris gel under non-reducing conditions at 200 V for 35 min using 2-(N-morpholino) ethanesulfonic acid (MES) running buffer (Invitrogen Corp., Carlsbad, California). The 12% Bis-tris gel was then fixed in 100 mL of 10% methanol/7% acetic acid for 15 min followed by overnight incubation in 50 mL Sypro Ruby (Molecular Probes, Eugene, OR) and finally rinsed in 100 mL of 10% methanol/7% acetic acid for 30 min. The protein staining on the gel was read at emission 488 nm and excitation 610 nm on a Fluorimager 595 with ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

1.8. Biological activity of released insulin

Insulin-loaded (6:3) hydrogels were incubated in serum free DMEM containing 5 mM glucose at 37 °C for 24 h. Based on FITC-insulin release kinetics, fresh serum free medium was added to the incubated medium to achieve a final insulin concentration of 10 nM. R-28 cells were grown in DMEM containing 5 mM glucose supplemented with 10% FBS and differentiated to neuron-like morphology on laminin-coated 60 mm dishes with addition of cell-permeable cAMP for 48 h. The cells were deprived of serum for 2 h and then treated with either free insulin or serum free DMEM incubated with insulin-loaded hydrogels at 10 nM for 5 min. The cells without insulin treatment served as controls. Western blotting was done for phosphotyrosine (PY) and beta subunit of insulin receptor (IR-β) as described previously [3].

1.9. In vivo safety assessment of hydrogels

Hydrogels were sterilized by UV irradiation for 20 min and implanted in the subconjunctival space in male Sprague-Dawley rats. All methods and care were in accordance with the Penn State Milton S. Hershey College of Medicine Institutional Animal Care and Use Committee guidelines and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were anesthetized with an intramuscular injection of a ketamine:xylazine (2:1) mixture at 0.6ml/kg. Proparacaine hydrochloride 0.5% drops were instilled into the eye to provide additional local anesthesia. The conjunctiva was cleaned with a cotton-tipped applicator soaked in povidine iodine 5% and immediately rinsed with sterile filtered 1X PBS. The superotemporal bulbar conjunctiva was grasped with a plain forceps and a radial incision was made using a pair of fine corneo-scleral scissors starting about 1mm posterior to the limbus and extending about 2 to 3 mm posteriorly. Any bleeding at this point was controlled by gentle pressure with cotton tipped applicators and rinsing with sterile filtered 1X PBS. The edge of the conjunctival incision was grasped with a plain forceps and blunt dissection was done in the subconjunctival space. A sterile dry hydrogel was grasped with a forceps and inserted into the subconjunctival space through the incision. The incision was closed with interrupted 9-0 vicryl sutures and antibiotic drops were instilled into the eye. Animals were euthanized after 7 days and tissue processed for histology and immunohistochemistry as previously described [18].

Full field electroretinograms (ERGs) were recorded simultaneously from both eyes of Sprague-Dawley (SD) rats (n=9) using a custom-built ganzfeld, a computer-based system (EPIC-XL, LKC Technologies, Inc, Gaithersburg, MD) and specially-made

contact lens electrodes (Hansen Ophthalmics, Iowa City, IA). Animals used for ERGs had implantation of insulin-loaded hydrogels (n=5) in one eye with the contralateral eye serving as a control with either blank hydrogel inserts (n=4) or by being untouched (n=1). Two other animals had a blank hydrogel insert in the study eye while the contralateral eye served as an untouched control. Age-matched, untouched, SD rats (n=6) and untouched eyes from the rats that received blank hydrogels (n=2) were used to define normal ranges. Details of our recording methodology in rats have been published [19]. In brief, animals were anesthetized with intramuscular injection of a mixture of ketamine HCl (75 mg/kg) and xylazine (5 mg/kg). Corneas were anesthetized with proparacaine HCl (0.5%) and pupils were dilated with tropicamide (1%) and phenylephrine (2.5%). Stimuli were delivered with medium energy (10 ms duration) and high energy (1 ms duration) flash stimulators with unattenuated white flash luminances of +0.8 and +3.6 log scot-cd.s.m⁻², respectively. Neutral density (Wratten 96, Kodak, Rochester, NY) and colored (Wratten 47A) filters were used to attenuate and spectrally shape the stimuli. Dark-adapted (>12 hrs) ERGs were obtained with blue stimuli increasing from -4.2 to -1.2 log scot-cd.s.m⁻² (averaging 2-8 responses), followed after a 1 min wait by a single flash (non-averaged) response to a +0.6 log scot-cd.s.m⁻² blue stimulus. After an additional 5 min wait, another single flash response was evoked with a +2.2 log scot-cd.s.m⁻² blue stimulus. A- and b-wave amplitudes were measured conventionally. Luminance-response functions were derived from b-wave series of study eyes (insulin-loaded hydrogels, n=5; blank hydrogels, n=2) and compared with normal ranges defined in untouched eyes (n=8). The interocular difference (IOD; left eye minus right eye) of b-wave amplitude evoked by a +0.6 log scot-cd.s.m⁻² blue flash was used to evaluate

differences between study eyes and controls. For this analysis, IOD limits (mean \pm 3SD) were defined in untouched rats (n = 4). Such an ERG IOD analysis has been previously used in mice [20], humans [21, 22], and non-human primates [23].

2. Results and discussion

2.1. Synthesis of degradable Poly(NIPAAm-co-Dex-lactateHEMA) hydrogels and FITC-insulin loading

In our previous work, we synthesized thermo-responsive and biodegradable hydrogels poly(NIPAAm-co-Dex-lactateHEMA) through thermal polymerization using potassium peroxydisulfate (KPS) as an initiator and N,N,N',N'-tetramethylethylenediamine (TEMED) as an accelerator [17]. In the present work, we synthesized similar hydrogels by UV polymerization using Irgacure 2959 solution as a photoinitiator to avoid the use of TEMED. The reason is that TEMED may be toxic *in vivo*, but Irgacure 2959 solution has been widely used for in situ polymerization *in vivo*, and does not cause eye irritation according to the Manufacturer's Safety and Data Sheet. The other advantage of this formulation is that drug can be loaded into the hydrogels during the synthesis process with high loading efficiency. For example, in this particular work, 15 wt% initially loaded FITC-insulin is 98% incorporated in the hydrogels after the synthesis. The parameters that we used for UV-synthesizing the Poly(NIPAAm-co-Dex-lactateHEMA) hydrogels are: DP (lactide chain length) and DS (number of lactide chains) of Dex-lactateHEMA macromer = 6 and 15, respectively; feeding weight ratios

NIPAAm/Dex-lactateHEMA=8/1, 6/3, and 4/5 (w/w); dimensions of the hydrogel disks: diameter = 2 and 4.5 mm and thickness =1.6 mm. The UV-synthesized hydrogels showed both thermoresponsive and degradable behavior similar to the previously reported hydrogels that were made using KPS and TEMED [17] (data not shown). The effects of the synthesis parameters on insulin release profiles from the hydrogels are studies in the following section.

3.2. In vitro insulin release from the hydrogels

First, we studied the effects of NIPAAm and Dex-lactateHEMA feeding weight ratios on FITC-insulin release profiles from the poly(NIPAAm-co-Dex-lactateHEMA) hydrogels (Fig. 1). The hydrogels with same DP=6 and DS=15 and dimension (2 mm in diameter and 1.6 mm in thickness) but different feeding weight ratios NIPAAm/Dex-lactateHEMA=8/1, 6/3, and 4/5 (w/w) can all release FITC-insulin for more than one week in PBS (pH=7.4) at 37 °C. The release rates increase with decreasing the NIPAAm and Dex-lactateHEMA feeding weight ratio in the order of 8/1 < 6/3 < 4/5 (w/w). Correspondingly, by using the power equation 1, we further calculated that the diffusion coefficients (k constant) of the three gels are 37.3, 42.9 and 53.8, respectively, which also increase with decreasing the NIPAAm and Dex-lactateHEMA feeding weight ratio (Table 1). The reasons may be that the hydrogels become more hydrophilic and degrade faster with increasing the feed weight ratio of hydrophilic Dex-lactateHEMA macromer/decreasing the feed weight ratio of NIPAAm monomer (which turns into hydrophobic polyNIPAAm at 37 °C) despite the fact that higher content of Dex-

lactateHEMA macromer may increase the crosslinking density of the hydrogels. The calculated n for the three gels are 0.27, 0.40 and 0.35 (Table 1) which are smaller than 0.5 suggesting that the insulin release is controlled by diffusion and the hydrophobicity of all the three gel networks increases with time due to degradation and diffusion of the Dex-lactateHEMA segment [17].

Second, we examined the effect of the dimension of poly(NIPAAm-co-Dex-lactateHEMA) hydrogels on the FITC-insulin release behavior (Fig. 1). Hydrogels with NIPAAm: Dex-lactateHEMA = 6/3 (w/w) feeding weight ratio and 1.6 mm thickness show less burst and slower release with larger diameter 4.5 mm than the ones with smaller diameter 2.0 mm. This result is also reflected by the calculated k constant using power equation 1, which is that the k value of the larger gels (20.4) is smaller than that of the smaller gels (40.9) as shown in Table 1. It is well known that if $0.5 < n < 1$ in power equation 1, drug release is controlled by both diffusion and swelling. Table 1 shows that the calculated n value of the larger gels (0.6) is higher than that of the smaller gels (0.40) indicating that the influence of the hydrogel swelling/degradation on the insulin release becomes stronger when the diameter of the hydrogels increases from 2 to 4.5 mm.

3.3. In vitro cytotoxicity of hydrogels to R28 retinal cells

In vitro MTT cytotoxicity studies of the synthesized blank hydrogels (2.0 mm in diameter and 1.6 mm in thickness) made of 6/3 feeding weight ratio of NIPAAm/Dex-lactateHEMA macromer demonstrate that no matter whether the hydrogels were incubated with R28 retinal cells directly for 24 h or they were degraded in the R28

culture medium for one week and then the cells were exposed with the degraded solution for 24 h, the viability of the treated cells is 100% of control values. These results suggest both the hydrogels and their degradation products are non-toxic to cultured R28 retinal cells for at least 1 and 7 days, respectively, and the hydrogels have potential for *in vivo* implantation.

3.4. Stability and biological activity of released insulin

Proteins are liable to degradation under unfavorable conditions, and a sustained delivery device must preserve the biological nature of proteins and prevent excessive degradation. We tested the stability of the insulin loaded into the hydrogels by incubating insulin-loaded hydrogels in 1mL PBS (pH 7.4) for 1 and 7 days and then running the PBS solutions on a non-denaturing gel as detailed in Materials and methods. Figure 2 illustrates a Sypro Ruby stained gel of the synthesized blank hydrogels (2.0 mm diameter and 1.6 mm thickness) made of 6/3 feeding weight ratio of NIPAAm/Dex-lactateHEMA macromer. The top band is from BSA that was used during encapsulation of insulin into the hydrogels. The bottom band represents insulin since it is the only other protein in the samples beside BSA. It is noted here that the molecular weight of the insulin band is slightly different from that of theoretical insulin because proteins that are not coated with SDS, in general, do not run according to their size during electrophoresis. There are no smear bands on the stained gel, so neither BSA nor insulin released from the hydrogels degraded during one week. Faint bands in the middle of the stained gel may represent dimeric or tetrameric forms of insulin.

To further test if the intact insulin released from the hydrogels also shows biological activity, we used Western blotting to measure the phosphotyrosine (PY) and beta subunit of insulin receptor (IR β) of R28 cells after incubated with the released insulin and free insulin at 10 nM for 5 min. Biologically active insulin binds to the α subunit of the insulin receptor and causes dimerization of the transmembrane β subunits. This in turn, leads to autophosphorylation of the tyrosine residues on the intracellular chain of β subunits. Autophosphorylation of the IR β triggers a series of phosphorylation events in the insulin signaling cascade which eventually lead to the biological effects of insulin. Figure 3 shows that insulin (10 nM) treatment for 5 min causes phosphorylation of the tyrosine residues of IR β , and 5 min incubation of serum-deprived R28 cells with medium containing insulin released from hydrogels induces phosphorylation of tyrosine residues of IR β to the same extent as 10 nM insulin. These data indicate that the hydrogels do not impair the biological effectiveness of the loaded insulin and hence may be used as a means of sustained insulin delivery to ocular tissues.

3.5. In vivo safety assessment of hydrogels

Hydrogels were implanted subconjunctivally in Sprague-Dawley rats as described in Materials and Methods. Figure 4 illustrates the relative size of the hydrogels and the rat eye (4a), and the rat eyes immediately (4b) and 7 days (4c) after subconjunctival implantation of the hydrogels. None of the rats showed any changes in feeding or behavior post implantation. After 7 days' implantation, the implanted eyes appear similar to the non-implanted eyes. To further evaluate if the implanted hydrogels caused any

morphological change and inflammation in the rat eyes, we performed H&E staining and immunostaining for Iba-1 in control, blank hydrogel implanted, and insulin-loaded hydrogel implanted eyes. The results show that hydrogel implanted eyes are not associated with a polymorphonuclear cell infiltrate or any morphological changes (Fig. 5), or increased Iba-1 expression, indicating absence of microglial cell activation indicative of inflammation (Fig. 6).

To assess the effect of the subconjunctivally implanted insulin delivery hydrogels on retinal physiology, full field bilateral simultaneous ERGs were analyzed in control, blank hydrogel implanted, and insulin-loaded hydrogel implanted eyes. Dark adapted ERGs in response to increasing intensities of light in two representative animals demonstrate that responses in implanted eyes, either with insulin-loaded hydrogels or blank hydrogels, are almost identical to those from the contralateral control (Fig. 7a). B-wave luminance-response functions from the five eyes implanted with insulin-loaded hydrogels show that four of five fall within normal limits (Fig. 7b) and the fifth eye has reduced b-wave amplitudes toward the higher intensity range of the function. Eyes carrying blank hydrogels are also within normal limits (data not shown). To assess better any possible effects of the insulin-loaded hydrogel implants on retinal electrophysiology, we examined responses elicited with medium energy stimuli ($+0.6 \log \text{ scot-cd.s.m}^{-2}$) in eyes implanted with insulin-loaded hydrogels compared to their contralateral eyes inserted with blank hydrogels (Fig. 7c). By inspection, three of four animals show nearly identical responses in both eyes, and the fourth implanted eye (animal #3635) shows smaller ERGs compared to the contralateral eye with a blank hydrogel insert. To determine the significance of the observed differences, ERG b-wave amplitudes in

response to the medium energy flash were measured conventionally in all implanted eyes (either insulin-loaded or blank) and plotted against their contralateral controls (blank or untouched). With the exception of the reduction in amplitude noted above in one animal, interocular differences (IODs) between eyes with insulin-loaded implants or blank implants and their contralateral controls did not exceed the limits determined in untouched animals (Fig. 7d). In summary, there was no consistent evidence of retinal toxicity by full field ERGs in eyes carrying insulin-loaded or blank hydrogels.

4. Conclusions

We have synthesized a series of water-based small dimensional hydrogel systems composed of NIPAAm monomer and Dex-lactateHEMA macromer using UV photopolymerization at room temperature. The hydrogels are both thermoresponsive and hydrolytically degradable and have diameters of 2 and 4.5 mm and thickness of 1.6 mm. All the hydrogels can successfully load insulin with up to 98% loading efficiency during the synthesis process. To the best of our knowledge, the designed hydrogels with 2 mm diameter are, to date, the smallest hydrogels formed in aqueous environment that can achieve one week of insulin release. The insulin release kinetics follow typical power law equation and apparently involve both diffusion and degradation mechanisms of the hydrogels. The dominating role of one of these two mechanisms on the insulin release is affected by the degree of crosslinking and hydrophilic/hydrophobic nature of the hydrogels which is determined by the ratio between NIPAAm and Dex-lactateHEMA macromer as well as the dimension of the hydrogels. The synthesized hydrogels and their

7-day degradable components are not toxic to R28 retinal cells *in vitro*. Moreover, subconjunctival implantation of the hydrogels did not cause any morphological change, inflammation or other adverse effects in the rat eyes over at least a one week period of time, as evidenced by the results of H&E staining, immunostaining for Iba-1, and ERGs. Therefore, the developed non-toxic hydrogels have high potential to control the release of insulin and other therapeutics to the retina after subconjunctival implantation, which may lead to a new option for treating diabetic retinopathy and other retinal disorders. The current ongoing work includes optimization of the hydrogel composition to achieve longer insulin release, studies on the ocular distribution of degraded hydrogel components and released insulin, and evaluations of the biological efficiency of released insulin in treating diabetic retinopathy.

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Figure Captions

Scheme 1. Schematic of Dex-lactateHEMA macromer. One lactide ring introduces two lactate spacer units after ring opening polymerization. DP is one lactate unit.

Fig. 1. Effects of NIPAAm and Dex-lactateHEMA feeding weight ratios and hydrogel dimensions on FITC-insulin release profiles from the poly(NIPAAm-co-Dex-lactateHEMA) hydrogels. The plots show time-dependent fractional release profiles of FITC-insulin from the hydrogels with dimension 2 mm diameter and 1.6 mm thickness and different feeding weight ratios NIPAAm/Dex-lactateHEMA (DP 6 and DS 15) = 8/1 (◆), 6/3 (●), and 4/5 (▲) and dimension 4.5 mm diameter and 1.6 mm thickness and feeding weight ratio NIPAAm: Dex-lactateHEMA (DP 6 and DS 15) = 6:3 (○) in PBS (pH 7.4) solvent at 37 °C. 15 wt% FITC-insulin and 10 wt% BSA were loaded into the hydrogels during the synthesis process. Solid lines are fitting curves based on Equation 1.

Fig. 2. Stability of insulin released from the synthesized hydrogels for 1 and 7 days. In the Sypro-ruby stained gel, Lanes 1 through 4 are 1X PBS samples incubated with insulin-loaded hydrogels at 37 °C for 24 h while lanes 5 through 8 are 7 day incubation samples. Hydrogels were made of 6/3 feeding weight ratio of NIPAAm/Dex-lactateHEMA macromer and have diameter and thickness in 2.0 and 1.6 mm, respectively.

Fig. 3. Biological activity of insulin released from the hydrogels using densitometric analysis of phosphotyrosine (PY) blot normalized to beta subunit of insulin receptor (IR β) of R28 cells after incubated with the released insulin and free insulin at 10 nM for 5 min. Hydrogels were made of 6/3 feeding weight ratio of NIPAAm/Dex-lactateHEMA macromer and have diameter and thickness in 2.0 and 1.6 mm, respectively. n is 2, 3 and 5 for control, 10nM insulin and hydrogel treated groups, respectively.

Fig. 4. Subconjunctival implantation of a hydrogel to the eye of a Sprague-Dawley rat. Hydrogels were made of 6/3 feeding weight ratio of NIPAAm/Dex-lactateHEMA macromer and have diameter and thickness in 2.0 and 1.6 mm, respectively.

Fig. 5. H & E stained sections from control, blank hydrogel implanted and insulin-loaded hydrogel implanted eyes. Hydrogel implanted eyes were not associated with an increased polymorpho-nuclear infiltrate or any morphological change. Hydrogels were made of 6/3 feeding weight ratio of NIPAAm/Dex-lactateHEMA macromer and have diameter and thickness in 2.0 and 1.6 mm, respectively. C – choroid, Pr –photoreceptor outer segments, ONL – outer nuclear layer, OPL – outer plexiform layer, INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer. Image magnification 200x.

Fig. 6. Iba-1 immunostained sections from control, blank hydrogel implanted and insulin-loaded hydrogel implanted eyes. There is no morphological change in the retina of hydrogel treated rat eyes. Microglia respond to inflammatory signals in the retina and Iba-1 is a microglial marker. Absence of altered immunostaining in hydrogel implanted

retina indicates the hydrogels do not induce an inflammatory response in the implanted eyes and are well tolerated. Hydrogels were made of 6/3 feeding weight ratio of NIPAAm/Dex-lactateHEMA macromer and have diameter and thickness in 2.0 and 1.6 mm, respectively. ONL–outer nuclear layer, OPL–outer plexiform layer, INL–inner nuclear layer, IPL–inner plexiform layer, GCL–ganglion cell layer. Image magnification 400X.

Fig. 7. Electroretinograms (ERGs) after subconjunctival implantation of insulin-loaded hydrogels in SD rats. (a) Dark adapted ERGs elicited by increasing intensities of light in implanted eyes (black traces: insulin-loaded or blank hydrogels) overlaid onto ERGs from contralateral untouched (thick gray traces) eyes. Traces start at stimulus onset; stimulus intensity is at the left of key traces; scale is at bottom right of waveforms; animal number top left of waveforms. ERGs from hydrogel-implanted eyes (insulin-loaded or blank) are very similar to their contralateral untouched controls. (b) B-wave luminance-response functions from all eyes with insulin-loaded hydrogels (open symbols). Gray band defines normal limits (mean \pm 3SD). Luminance-response functions in implanted eyes fall within the normal range in all but one animal. (c) ERGs in response to medium-energy light stimuli in four animals implanted with insulin-loaded hydrogels compared to their contralateral eye implanted with blank hydrogels. With the exception of one animal, responses in insulin-loaded implanted eyes are nearly identical to eyes with blank implants. (d) B-wave amplitude in implanted eyes (insulin-loaded or blank) plotted against contralateral controls (blank or untouched). Dashed line represents no inter-ocular difference in this parameter. Limits of significant inter-ocular difference

derived from untouched animals are shown a with solid gray line. All but one of the implanted eyes fall within IOD limits. Hydrogels were made of 6/3 feeding weight ratio of NIPAAm/Dex-lactateHEMA macromer and have diameter and thickness of 2.0 and 1.6 mm, respectively.

Table 1. Fitting parameters in Equation 1 for the release kinetics of hydrogels in PBS (pH 7.4) at 37 °C.

Hydrogel Samples	NIPAAm/Dex-lactateHEMA feeding weight ratios	Diameter mm	Thickness mm	k	n	R^2
Gel 1	8/1	2.0	1.6	37.3	0.27	0.990
Gel 2	6/3	2.0	1.6	42.9	0.40	0.986
Gel 3	4/5	2.0	1.6	53.8	0.35	0.978
Gel 4	6/3	4.5	1.6	20.4	0.60	0.993

Scheme 1

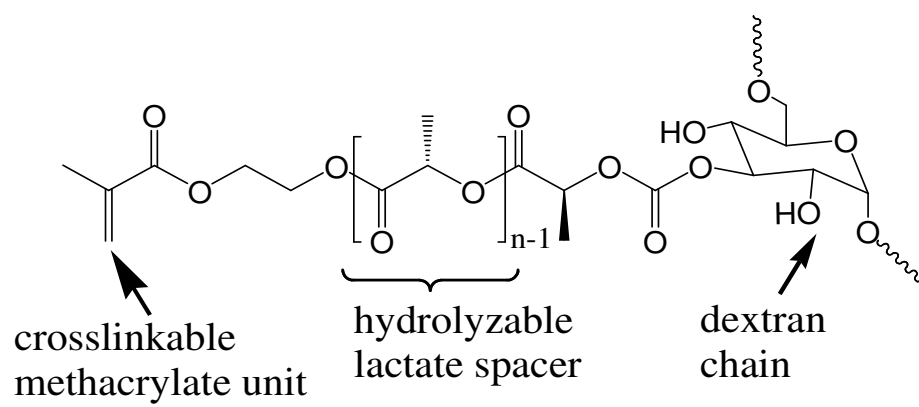


Fig. 1

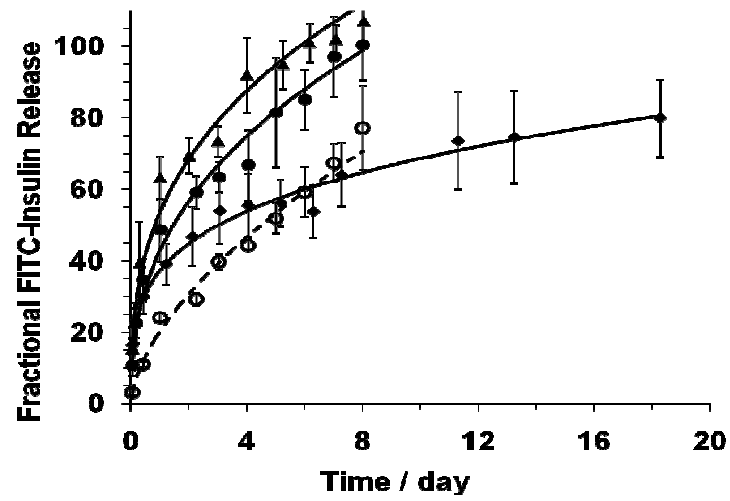


Fig. 2

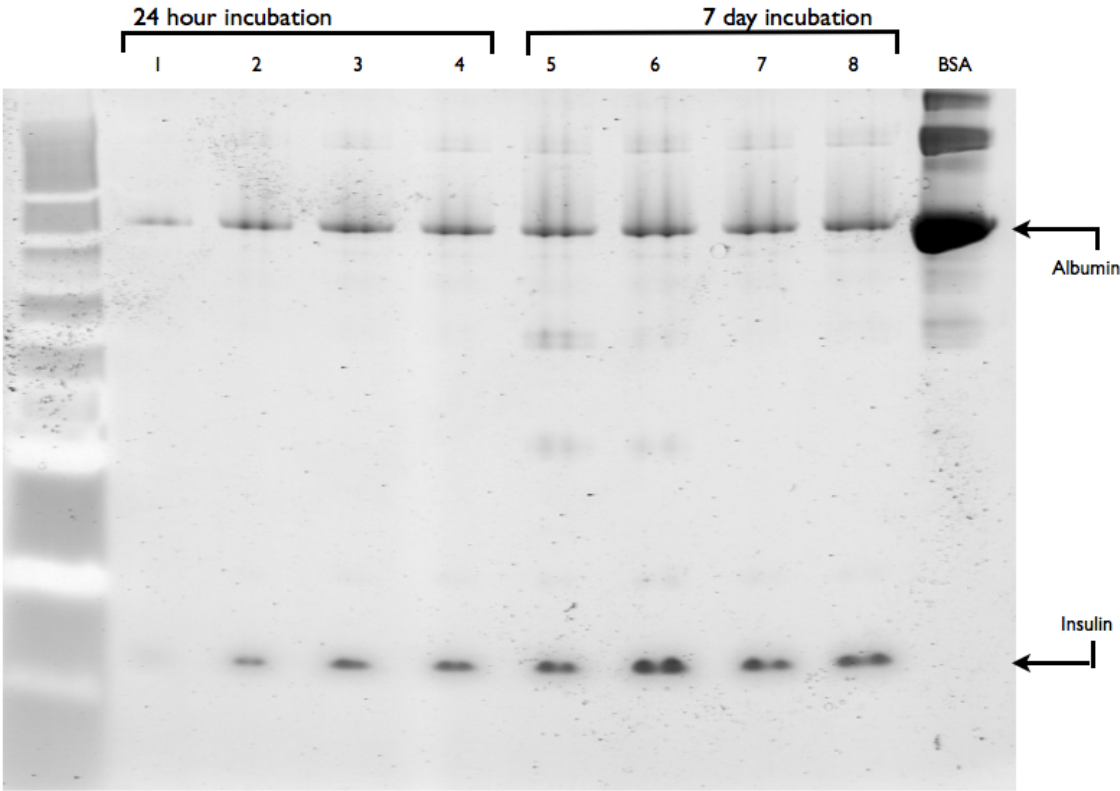


Fig. 3

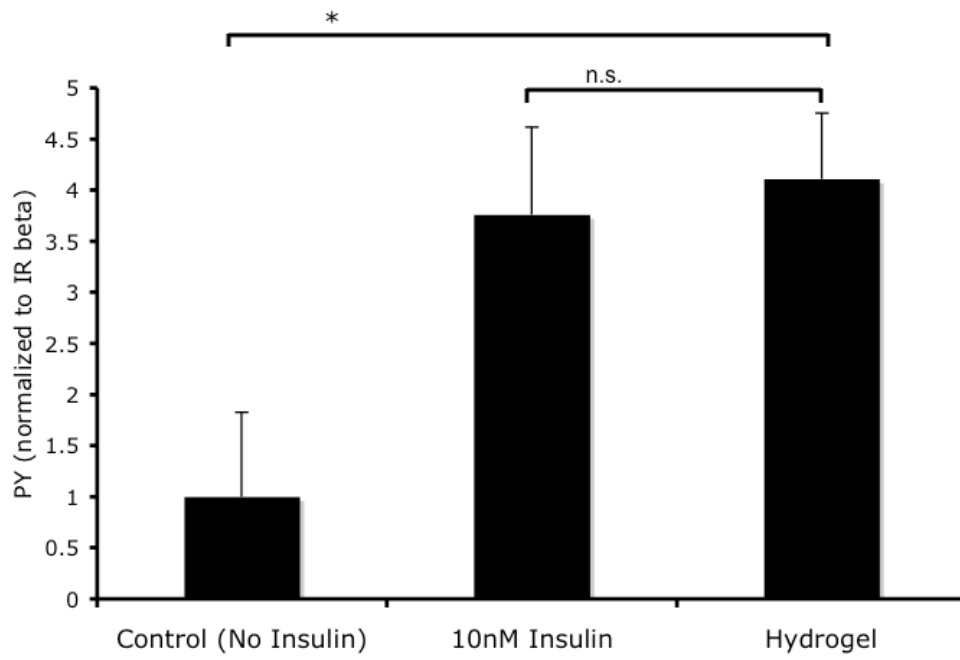


Fig. 4



Hydrogel and Rat's eye



Hydrogel Implanted under
conjunctiva



Rat's Eye 1 week after
implantation

Fig. 5

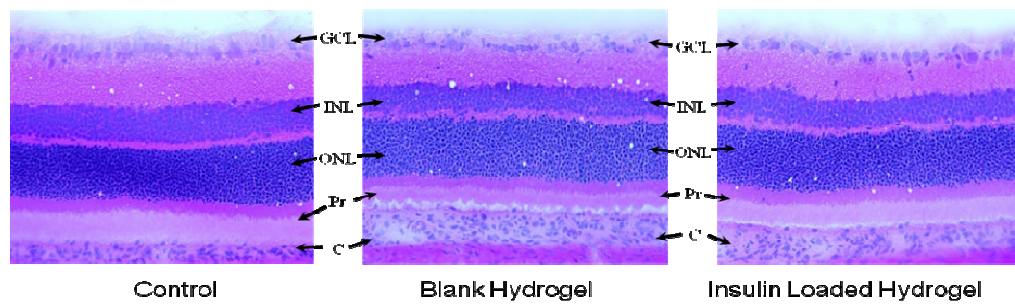


Fig. 6

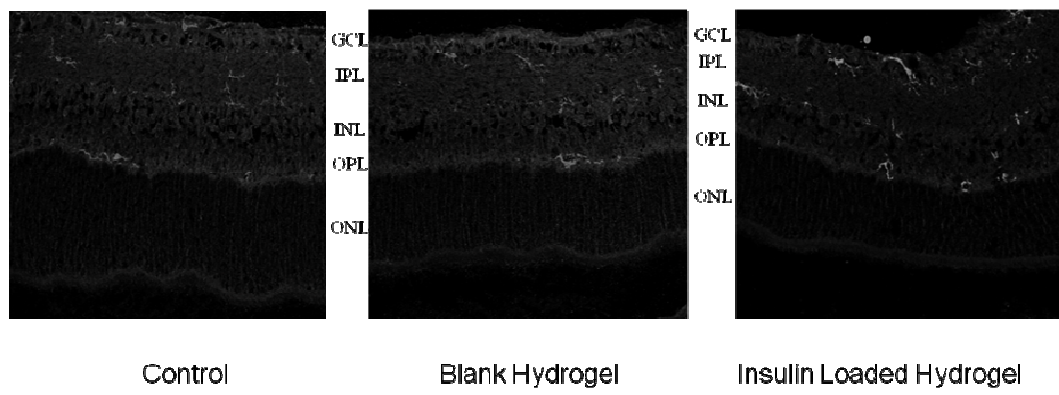


Fig. 7

