Subconjunctivally Implanted Hydrogels for Sustained Insulin Release to Reduce Retinal Cell Apoptosis in Diabetic Rats

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PURPOSE. Diabetic retinopathy (DR) is a leading cause of blindness in diabetic patients that involves early-onset retinal cell loss. Here, we report our recent work using subconjunctivally implantable hydrogels for sustained insulin release to the retina to prevent retinal degeneration.

METHODS. The hydrogels are synthesized by UV photopolymerization of N-isopropylacrylamide and a dextran macromer containing oligolactate-(2-hydroxyethyl methacrylate) units. Insulin was loaded into the hydrogels during the synthesis. The ex vivo bioactivity of insulin released from the hydrogels was tested on fresh rat retinas using immunoprecipitation and immunoblotting to measure insulin receptor tyrosine and Akt phosphorylation. The biosafety and the effect on the blood glucose of the hydrogels were evaluated in rats 2 months after subconjunctival implantation. The release of insulin from the hydrogels was studied both in vitro in PBS (pH 7.4), and in vivo using confocal microscopy and RIA kit. The in vivo bioactivity of the released insulin was investigated in diabetic rats using DNA fragmentation method.

RESULTS. The hydrogels could load insulin with approximately 98% encapsulation efficiency and continuously release FITC-insulin in PBS (pH = 7.4) at 37°C for at least 5 months depending on their composition. Insulin lispro released from the hydrogels was biologically active by increasing insulin receptor tyrosine and Akt serine phosphorylation of ex vivo retinas. In vivo studies showed normal retinal histology 2 months post subconjunctival implantation. Insulin released from subconjunctivally implanted hydrogels could be detected in the retina by using confocal microscopy and RIA kit for 1 week. The implanted hydrogels with insulin lispro did not change the blood glucose level of normal and diabetic rats, but significantly reduced the DNA fragmentation of diabetic retinas for 1 week.

CONCLUSIONS. The developed hydrogels have great potential to sustain release of insulin to the retina via subconjunctival implantation to minimize DR without the risk of hypoglycemia.

Keywords: diabetic retinopathy, insulin, hydrogels, subconjunctival implantation, DNA fragmentation

Diabetic retinopathy (DR) affects 14% of the 29 million Americans with diabetes aged 20 to 74 years.¹ Currently laser surgery and vitrectomy are used to treat advanced DR, but they are destructive and do not address the underlying biology of DR.² Inhibition of VEGF reduces macular edema,³ but does not support the integrity or loss of retinal neurons that die by apoptosis.^{4–10} Specifically, neurons (photoreceptors, bipolar cells, horizontal cells, amacrine cells, and ganglion cells) and glia (astrocytes, Müller cells, and microglial cells) comprising the retina are impaired in DR. To target the neuronal degeneration in DR, insulin has been investigated as a potential therapeutic to rescue retinal neurons from apoptosis.^{11–13} Our previous work showed that the retina exhibits high basal activity of the insulin receptor/phosphoinositide 3-kinase/Akt signaling system,¹⁴ and short-term experimental diabetes in rats reduces

this activity.¹¹ Systemic or intravitreally injected insulin restores prosurvival insulin receptor and Akt kinases and rescues retinal neuronal cells from apoptotic cell death,^{12,15} and hyperglycemia abrogates this effect.^{12,16} Moreover, intensive systemic insulin therapy reduces the risk of retinopathy onset and progression, and retinopathy is associated with insulin resistance.^{17,18} Together these results demonstrate that insulin receptor stimulation may have therapeutic implications for DR.

Insulin has a short half-life in plasma, and its long-acting analogues such as insulin glargine and insulin degludec have durations of action only up to 24 hours.^{19,20} The risk of hypoglycemia also limits the ability of patients to take enough insulin systemically to minimize the risk of retinopathy.²¹ Intravitreal injection of insulin can be an effective route to deliver drugs to the retina compared with topical and

FIGURE 1. Scheme of Dex-lactateHEMA macromer (DP = n here).

subconjunctival routes, but intravitreal injection is invasive, and can rarely cause endophthalmitis.^{22,23} Eye drops are the most commonly used delivery form in ocular drug treatment, but they are neither patient compliance nor efficient for the treatment of diseases of the posterior segment of the eye.²⁴ Factors that limit the usefulness of eye drops include corneal and conjunctival barriers, and the rapid and extensive precorneal tear loss due to drainage and tear fluid turnover.²⁵ The subconjunctival space external to the sclera has received increasing attention as a minimally invasive and effective route for delivering drugs to the retina, obviating the risks of intraocular surgery and systemic side effects.^{26–28} Subconjunctivally injected very low-dose insulin activates prosurvival insulin receptor signaling pathways in the treated eye without lowering blood glucose or affecting the opposite eye.^{29,30} Taken together, controlled and sustained release of biologically active therapeutics to activate the retinal insulin receptor via a subconjunctival route may be clinically beneficial.

We previously reported the design of subconjunctivally implantable hydrogels for insulin delivery to the retina.³¹ We demonstrated that the hydrogels are nontoxic *in vitro* to retinal neuronal cells and *in vivo* in rats, and capable of release insulin for more than 1 week *in vitro*. In this work, we improved the release property of the hydrogels and evaluated their potential for managing DR via subconjunctival implantation. The hydrogels were synthesized via UV polymerization with a slight modification from our previous report³¹ in terms of the solvent used, and the average repeating lactate units (DP) in the cross-linkable chains of the macromers used. The *ex vivo* bioactivity of the insulin released from the hydrogels was tested on freshly extracted retinas from rats using immunoprecipitation and immunoblotting to measure insulin receptor (IR) tyrosine and Akt phosphorylation. The biosafety of the hydrogels was evaluated in rats 2 months post implantation using hematoxylin-eosin (H&E) staining. The release of insulin from the hydrogels was studied both *in vitro* using PBS (pH 7.4) medium, and *in vivo* using confocal microscopy and radioimmunoassay (RIA) kit after 1 week subconjunctival implantation in rats. The effect of the subconjunctivally implanted hydrogels on the blood glucose level of rats was monitored. The *in vivo* bioactivity of insulin released from the subconjunctivally implanted hydrogels was investigated in diabetic rats by using the DNA fragmentation method.

MATERIALS AND METHODS

Materials

Dextran ($M_w = 15,000$ g·mol⁻¹) was purchased from Polysciences, Inc. (Warrington, PA, USA). The following materials were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA): N-isopropylacrylamide (NIPAAm), 2-hydroxyl methacrylate (HEMA), 4-(N,N-diethylamino) pyridine (DMAP), N,N'-carbonyl-diimidazole (CDI), L-lactide, stannous octoate (SnOct₂), tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), dimethylformamide (DMF), fluorescein isothiocyanate (FITC), mini-

TABLE 1. Composition for Hydrogel Synthesis

Hydrogel Sample	Feeding Ratio		
	NIPAAm, mg	Dex-LactateHEMA, mg	Solvent DMF, μ L
G-8-1	8	1	18
G-6-3	6	3	18
G-4-5	4	5	18

um essential medium (MEM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and streptozotocin (STZ). All the chemicals were used as received except that the solvents used in the synthesis of Dex-lactateHEMA macromeres were treated with 4A molecular sieves for at least 1 day. Deionized distilled water was used in all the experiments.

Insulin lispro was extracted from Humalog (insulin lispro injection, 100 IU·mL⁻¹, potency 27 IU·mg⁻¹, Eli Lilly and Company (Indianapolis, IN, USA) by dialysis. The FITC-insulin was synthesized from insulin lispro according to our previous publication.³¹ All antibodies were purchased and used according to the manufacturer's suggestions. Anti-insulin receptor β (anti-IR β) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-phosphotyrosine (PY) was from Upstate Biotechnology, Inc. (Lake Placid, NY, USA), and anti-phospho^{Ser473} and anti-total Akt were from Cell Signaling Technology (Beverly, MA, USA). Sensitive rat RIA kit (#SRF-13K) was from Millipore (St. Charles, MO, USA). The DC protein assay kit was from Bio-Rad (Hercules, CA, USA). Enzyme-linked immunosorbent assay (ELISA) kit for DNA fragmentation study was from Roche Diagnostics GmbH (Mannheim, Germany).

Synthesis of Hydrogels

Dextran macromers containing oligolactate-(2-hydroxyethyl methacrylate) units (Dex-lactateHEMA, Fig. 1) with DP = 8 (the lactide chain length of the lactate unit) and DS = 15 (the average number of cross-linkable chains per dextran) were synthesized using the procedure described previously^{32,33} with the feeding ratios of precursors modified accordingly. The DP and DS of the macromers were estimated from the ¹H NMR spectra. Poly(NIPAAm-co-Dex-lactateHEMA) hydrogels (Table 1) loaded with FITC-insulin or insulin lispro were synthesized using UV-initiated free radical polymerization method in a Teflon mold with well-defined wells (2 mm in diameter and 1.6 mm in height). To synthesize FITC-insulin-loaded hydrogels, a first type of pre-polymer solutions was prepared from NIPAAm/Dex-lactateHEMA with weight ratios of 8/1 (for hydrogels G-8-1), 6/3 (for hydrogels G-6-3), or 4/5 (for hydrogels G-4-5), and 5 wt% FITC-insulin in 0.1 wt% Irgacure 2959 in DMF; 2 μ L of solvent was used for each milligram of the total weight of NIPAAm and Dex-lactateHEMA macromer (Table 1). To synthesize insulin lispro-loaded hydrogel discs, a second type of pre-polymer solutions was prepared by replacing the FITC-insulin with 6.4 IU/gel insulin lispro in the first type of pre-polymer solutions. In addition to synthesizing blank hydrogel discs, a third type of pre-polymer solutions was prepared by not including FITC-insulin in the first type of pre-polymer solutions. The pre-polymer solutions were added into the Teflon mold and then exposed to 500 mW/cm² UV light EXFO Lite UV source (EXFO, Inc., Richardson, TX, USA) for 3 minutes. The resulting hydrogels were carefully peeled out from the well and washed with deionized water (DI) twice. The fluorescent intensity of the wash solutions was measured and the amount of insulin in the wash solutions was

determined from a standard curve. The insulin encapsulation efficiency was calculated by subtracting the amount of insulin in the wash solutions from the originally loaded insulin amount and then dividing by the originally loaded insulin amount. The result showed that the encapsulation efficiency of all the hydrogels was approximately 98%: $97.64\% \pm 0.79\%$, $98.64\% \pm 0.31\%$, and $98.41\% \pm 0.04\%$ for hydrogels G-8-1, G-6-3, and G-4-5, respectively. The loading content of the hydrogels was calculated to be approximately 4.7%: $4.65\% \pm 0.09\%$, $4.67\% \pm 0.27\%$, and $4.72\% \pm 0.39\%$ for hydrogels G-8-1, G-6-3, and G-4-5, respectively. The obtained hydrogel discs were frozen with liquid nitrogen and dried in a freeze-dryer overnight. The hydrogel discs without insulin are referred to as “blank hydrogels,” those with FITC-insulin are referred to as “FITC-insulin-loaded hydrogels,” and those with insulin lispro are referred to as “insulin lispro-loaded hydrogels” in the subsequent sections.

In Vitro Insulin Release From Hydrogels

The FITC-insulin-loaded hydrogels were placed into glass vials and then 1 mL PBS (pH 7.4) was added into the vials in a 37°C shaking water bath. At prescheduled time points, 50- μ L aliquots were withdrawn from the vials and an equal volume of PBS (pH 7.4) was added to compensate for the released medium. Fluorescence intensity of the samples was quantified using a SPECTRAMax GEMINI fluorescence plate reader (Molecular Devices, LLC., Sunnyvale, CA, USA) and the concentrations of FITC-insulin in the samples were determined using a calibration curve that was $y = 26167x$, $R^2 = 0.9997$. The amounts of insulin released from the hydrogels were calculated from these concentrations accordingly.

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 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, whereas values higher than 0.5 are regarded as anomalous diffusion. A zero order release is obtained if n equals 1.

Animals and Animal Care

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA), 200–350 g, were used and age-matched in all studies. Unless otherwise noted, the rats used were normal rats without diabetes. All methods and care related with animals were performed 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.

Ex Vivo Bioactivity of Insulin Released From Hydrogels

An ex vivo retina tissue system was used to evaluate the IR tyrosine and Akt phosphorylation in response to insulin lispro-loaded hydrogel G-6-3, as described previously.^{11,14,30,34} Briefly, retinas were removed from rats and each retina was cultured in 50 μ L MEM medium supplemented with 5 mM pyruvate and 10 mM HEPES at 37°C, 5% CO₂ with gentle shaking. The G-6-3 hydrogel discs with and without insulin lispro were added by immersing one hydrogel disc together with one retina in the 50 μ L medium for 5, 15, and 30 minutes ($n = 4$ per group per time

point). Retinas were homogenized by sonication in lysis buffer and 500 μ g of tissue lysates were immunoprecipitated with anti-IR β antibody and immunoblotted with anti-phosphotyrosine (PY) antibody. The ratio of PY to IR β was calculated. The Akt activity was determined by the ratio of Akt^{Ser473} phosphorylation to total Akt after immunoblotting the retinal lysates with antiphospho^{Ser473} and anti-Akt antibodies. Retinas incubated in the medium without the hydrogels and with 100 nM insulin lispro were used as negative and positive controls, respectively.

In Vivo Safety and Bioefficacy of Insulin-Loaded Hydrogels

Subconjunctival Implantation of Hydrogels. Freeze-dried blank or insulin-loaded G-6-3 hydrogel discs were sterilized using UV irradiation for 20 minutes and implanted in the subconjunctival space of the right eyes of male Sprague-Dawley rats according to the previously described procedure.³¹ Briefly, a 1- to 2-mm radial incision was made posterior to the limbus of the anesthetized rat, and a sterile dry G-6-3 hydrogel was 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.

In Vivo Biosafety of Subconjunctivally Implanted Hydrogels. Four blank G-6-3 hydrogels were subconjunctivally implanted into the right eyes of Sprague-Dawley rats (one hydrogel/eye) to investigate the biosafety of the hydrogels. Rats were euthanized after 2 months and eyeballs were enucleated to extract retinas. Histology and immunohistochemistry studies were performed by H&E staining as previously described.^{31,35}

In Vivo Insulin Release From Hydrogels: Detection of Insulin in the Retina After Subconjunctival Implantation of Insulin-Loaded Hydrogels. Twelve FITC-insulin-loaded G-6-3 hydrogels were implanted subconjunctivally into the right eyes of Sprague-Dawley rats (one hydrogel/eye) to visualize the delivery of insulin from the hydrogels to the retina. Four of the 12 rats were euthanized at each of three time points: 1 day, 1 week, and 1 month after the implantation, and the retinas receiving the hydrogel implantation were removed as previously described.¹⁴ Hoechst staining was performed on the extracted retinas, and retinal images were taken by confocal microscopy.

In addition, eight insulin lispro-loaded G-6-3 hydrogels were also implanted subconjunctivally into the right eyes of Sprague-Dawley rats (one hydrogel/eye). Blood samples were collected before half of the eight rats were euthanized at each of two time points: 1 day and 1 week post implantation and eyes were enucleated to extract the retinas. Serum and retinal insulin levels were measured using a sensitive rat RIA kit (#SRI-13K) as previously described.³⁰ Briefly, serum was obtained by promptly centrifuging the clotted blood at 2000g for 15 minutes at $4 \pm 2^\circ\text{C}$. Each retina was sonicated in 120 μ L of assay buffer supplied in the RIA kit with protease inhibitor, and lysates were rocked at 4°C for 15 minutes followed by centrifugation at 10,000g for 10 minutes. The supernatant was taken out to detect insulin concentration using the RIA kit. Protein concentrations of tissue lysates were determined using a DC protein assay kit and retinal insulin levels were normalized to total retinal protein concentration.

Effect of Insulin Released From Hydrogels on DNA Fragmentation in Diabetic Retinas After Subconjunctival Implantation. Experimental type 1 diabetic rats were induced by intraperitoneal injection of streptozotocin (STZ) (65 mg/kg, 0.24–0.28 mL) in 10 mM sodium citrate buffer (pH 4.5) into normal rats after 7 days of acclimation and an overnight fast. Nondiabetic age-matched control rats

FIGURE 2. Cumulative release of FITC-insulin from poly(NIPAAm-co-Dex-lactateHEMA) hydrogels G-8-1 (◆), G-6-3 (●), and G-4-5 (▲) in vitro at 37°C ($n = 4$).

received equivalent volumes of buffer alone. The STZ-injected rats were considered diabetic when exhibiting blood glucose levels higher than 13.9 mM (250 mg·dL⁻¹) within 5 days after diabetes induction (One-Touch meter; LifeScan, Milpitas, CA, USA). The rats had free access to a standard rat chow and water, and were housed under a 12-hour light/dark cycle.

Eight weeks later after diabetes induction, either 12 insulin lispro-loaded G-6-3 hydrogels or 8 blank G-6-3 hydrogels were implanted into the right eyes of diabetic rats (one hydrogel/eye), and 16 normal and 8 diabetic rats without implantations were used as controls. Half of each type of the treated or untreated retinas were extracted at each of two time points: 2 days and 1 week post implantation and used for the quantification of treatment effect by a cell death detection ELISA to detect fragmented retinal DNA, indicative of late-stage apoptosis. The assay was performed according to the manufacturer's instructions with minor modifications. Briefly, the extracted retina was homogenized in 200 μ L lysis buffer and then centrifuged at 10,000g at 4°C for 15 minutes; 20 μ L of the supernatant, as well as of the positive and negative controls were added into separate wells of the ELISA plate, followed by addition of 80 μ L of immunoreagent into the each well. The ELISA plate was shaken at 300 rpm on an orbital rocker at room temperature for 2 hours. The supernatant in each well was removed and the wells were washed three times with incubation buffer; 100 μ L of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) colorimetric solution was added into each well and incubated on an orbital rocker at 250 rpm at room temperature for 15 minutes. Stop solution was added, and the colorimetric signal was measured with a fluorescence plate reader (SpectraMax GEMINI EM; Molecular Devices) with excitation at 405 and 490 nm. The DNA fragmentation was quantified by subtracting the optical density of the negative control from that of the sample and normalized by retinal protein concentration. Body blood glucose levels in normal control rats and normal rats subconjunctivally implanted with blank and insulin lispro-loaded G-6-3 hydrogels were monitored before implantation and 1 day, 1 week, 1 month, and 2 months after hydrogel implantation ($n = 4$ per group per time point).

Statistical Methods

Data were reported as mean $\pm$ SD or mean $\pm$ SE from at least three separate experiments. Two-tailed Student's *t*-test and Bonferroni post hoc test were used to analyze the differences between treatment groups. A statistically significant difference was reported if $P = 0.05$ or less.

RESULTS AND DISCUSSION

In Vitro Insulin Release

In previous work we demonstrated that poly(NIPAAm-co-Dex-lactateHEMA) synthesized by UV polymerization could release FITC-insulin for 1 to 3 weeks and the release duration was affected by the size of the hydrogel and also the feeding ratios of NIPAAm to Dex-lactateHEMA macromers.³¹ To make the hydrogels release insulin longer, we modified the Dex-lactateHEMA macromer by changing the DP from 6 to 8 and used DMF instead of ethanol/water (3:7) as solvent for the hydrogel synthesis. Figure 2 shows the in vitro FITC-insulin release profiles from three different hydrogels: G-8-1, G-6-3, and G-4-5. All three types of hydrogels could continuously release FITC-insulin in vitro for more than 5 months, demonstrating that the hydrogels synthesized in this work significantly increased insulin release duration than the previously reported hydrogels.³¹ The hydrogels G-4-5 showed bimodal release: fast release during the first month and slow release afterward. The first-phase fast release was probably due to the fast degradation of Dex-lactateHEMA in the beginning and the second-phase slow release was probably because the degradation of the hydrogels became slower as more hydrophilic dextran chains diffused out after the hydrolytic degradation and the hydrogels became more hydrophobic with time. The cumulative amount of insulin released during the slow-release period increased from approximately 17% on day 28 to approximately 23% on day 155, which was very slow but the change was significant, and the release amount was able to be detected at each time point when samples were collected. The hydrogels G-6-3 released FITC-insulin slower than the hydrogels G-4-5 but faster than the hydrogels G-8-1 (G-8-1 < G-6-3 < G-4-5), suggesting that the release rate of FITC-insulin decreased with increasing the weight ratio of NIPAAm to Dex-lactateHEMA in the hydrogels, which was consistent with our previous observation.³¹ As noted previously, this result might be because at 37°C the hydrophobicity of PNIPAAm component had more effect on the FITC-insulin release kinetics than the crosslinking density caused by the Dex-lactateHEMA component in the hydrogels.³¹ It is worthy to note that even though insulin was released from the hydrogels G-6-3 and G-8-1 slowly with time, the amount of insulin released at each time point was detectable and significant.

The FITC-insulin in vitro release data were further analyzed by using a power Equation 1^{36,37} with the fitting results summarized in Table 2. Table 2 shows that the constant *k* values of the hydrogels G-8-1, G-6-3, and G-4-5 were in the range of approximately 1 to 5 day^{-*n*} (*n* is the exponent in Equation 1), which were significantly lower than those in the range of approximately 35 to 55 day^{-*n*} of the corresponding hydrogels previously reported.³¹ However, the *n* values of the hydrogels G-8-1, G-6-3, and G-4-5 were 0.26, 0.41, and 0.33, respectively, which were similar to the respective *n* values 0.27, 0.4, and 0.35 of the corresponding hydrogels previously reported with the same feeding ratios of NIPAAm to Dex-lactateHEMA.³¹ These results imply that the slight increase of DP in the Dex-lactateHEMA macromers from 6 to 8 and the change of solvent from ethanol/water

TABLE 2. Fitting Parameters in Equation 1 for the Release of FITC-Insulin From Hydrogels in PBS (pH 7.4) at 37°C

Hydrogel Samples	Diameter, mm	Thickness, mm	k, d ⁻ⁿ	n	R ²	k, d ⁻ⁿ	n
G-8-1	2	1.6	1.35	0.26	0.990	37.3*	0.27*
G-6-3	2	1.6	1.11	0.41	0.890	42.9†	0.4†
G-4-5	2	1.6	4.2	0.33	0.952	53.8‡	0.35‡

*, †, ‡, reported in our previous paper for insulin release from poly(NIPAm-dex-lactateHeMA) with feeding ratio of 8:1, 6:3 and 4:5 for NIPAm to Dex-lactateHEMA (DP=6 and DS=15), respectively.

(3:7) to DMF for the hydrogel synthesis significantly decreased the diffusion rate of FITC-insulin from the hydrogels because the two changes made the hydrogels more hydrophobic. However, the two changes did not alter the FITC-insulin transport mechanism from the hydrogels, which was diffusion-controlled transport mechanism with the hydrophobicity of all the hydrogel networks increasing with time due to degradation and diffusion of the Dex-lactateHEMA segment.^{31,32}

The purpose of this work was to provide long-term local insulin therapy in the retina to treat DR without increasing insulin plasma concentration to cause hypoglycemia. In our previous study, we found that subconjunctivally injected very low-dose insulin of approximately 0.01 to 1.4 µg activated prosurvival IR signaling pathways to potentially normalize diabetes-induced retinal abnormality and prevent vision loss without affecting blood glucose levels in rats.^{29,30} Figure 2 shows that the hydrogels G-6-3 steadily released

approximately 0.2 to 0.4 µg insulin falling in the approximately 0.01 to 1.4 µg effective low-dose insulin range for 5 months in a near zero-order release fashion, and thus hydrogels G-6-3 were chosen for the further ex vivo and in vivo studies.

Ex Vivo Bioactivity of Insulin Released From Hydrogels

We previously reported that insulin could rescue retinal neurons from apoptosis after inducing IRβ tyrosine and Akt phosphorylation.^{11–14,30,38} However, proteins are generally unstable, especially in contact with organic solvents and after long-term storage at elevated temperature. To rescue retinal cells from apoptosis, the insulin released from the hydrogels must be stable and maintain its bioactivity. To evaluate these features, we incubated freshly extracted retinas with hydrogels with and without insulin lispro in tissue culture medium for 0, 5, 15, and 30 minutes; homogenized the retinas; and performed immunoprecipitation and immunoblotting to measure IRβ tyrosine and Akt phosphorylation. Retinas incubated in the medium without the hydrogels and with 100 nM insulin lispro were used as negative and positive controls, respectively. Figure 3 shows that the hydrogels G-6-3 did not impair IRβ tyrosine and Akt phosphorylation in the retina. However, the insulin lispro loaded G-6-3 hydrogels induced significant increases of the two kinases to the same extent as 100 nM insulin during the 30-minute incubation time, and caused the highest phosphorylation of the two kinases at 5-minute incubation time.

FIGURE 3. Effects of insulin lispro released from G-6-3 hydrogels on the IRβ tyrosine and Akt phosphorylation in rat retinas ex vivo. The IR from retina was immunoprecipitated and analyzed for tyrosine phosphorylation (PY) of the IR and Akt kinase activity against time during 30-minute incubation time. (A) Representative PY and IRβ blots and fold increase of PY/IRβ ratios; and (B) representative Akt^{ser473} and Akt^{total} blots and fold increase of Akt^{ser473}/Akt^{total} ratios. (○) control, (△) blank hydrogels, (◇) hydrogels G-6-3, and (□) 100 nM insulin-positive control. For all the ratios, time 0 was set to be 1. *n* = 4 per group per time point; ***P* < 0.01 from time 0 by ANOVA and Bonferroni post hoc test.

FIGURE 4. Retinal histology of rat receiving subconjunctival implantation of blank G-6-3 hydrogels (H&E staining, $\times 40$). Hydrogel-implanted eyes were not associated with an increased polymorphonuclear infiltrate or any morphological change during 2-month implantation. $n = 4$.

In Vivo Biosafety of Subconjunctivally Implanted Hydrogels

We previously showed the lack of *in vitro* cytotoxicity of the hydrogels synthesized in ethanol/water from 6:3 w:w feeding ratio of the NIPAAm monomer to Dex-lactate/HEMA macromer to R28 retinal cells.³¹ Moreover, subconjunctival implantation of the hydrogels did not cause any morphological change, inflammation, or other adverse effects in rat eyes over at least one week, as evidenced by the results of H&E staining and immunostaining for Iba-1 and ERGs. Because we switched the solvent from ethanol/water to DMF for the synthesis of the hydrogels in the present study, we needed to reconfirm that the resulting hydrogels were safe for subconjunctival implantation. Therefore, blank G-6-3 hydrogels were implanted into rat eyes and the retinas showed no evidence of inflammation or neurodegeneration 2 months after the implantation (Fig. 4). This result indicates that the hydrogels synthesized in this study were well tolerated by rats.

In Vivo Insulin Release From Hydrogels

With the above data demonstrating that the hydrogels were well tolerated *in vivo*, the next question was if the hydrogels could deliver insulin to the retina after subconjunctival implantation. Figure 5 shows the confocal images of retinas

FIGURE 5. Confocal images of insulin in the retinas of rat eyes receiving subconjunctival implantation of FITC-insulin-loaded G-6-3 hydrogels for 1 month. Retinal sections 10- μ m thick were used for imaging. Shown are representative photomicrographs after 1-day, 1-week, and 1-month implantation. In each vertical panel, the *top*, *middle*, and *bottom* sections were the retinas treated with nothing, blank hydrogels, and FITC-insulin loaded hydrogels at each time point. The *blue* color came from the DAPI-stained nuclei and the *green* color came from the FITC-labeled insulin. The white bar represents 100 μ m. $n = 4$ per group per time point.

FIGURE 6. Blood glucose level in normal control rats ($\times$), and normal rats subconjunctivally implanted with blank G-6-3 hydrogels ($\triangle$), and insulin lispro-loaded G-6-3 hydrogels ($\square$) for 0 (preimplantation), 1 day, 1 week, and 1 and 2 months. $n = 4$ per group per time point.

extracted from the rat eyes receiving implantation of FITC-insulin-loaded G-6-3 hydrogels for 1 day, 1 week, and 1 month. The FITC-insulin was detected in all the retinas of rats receiving FITC-insulin implantation but not in the control retinas. The fluorescence intensity at day 1 was somewhat stronger than that at week 1, and much greater than at month 1. These observations were confirmed by the insulin RIA results showing that 12.24 ± 1.26 , 7.13 ± 1.74 and 0 μ IU insulin/mg total retinal protein were detected in the retina after the subconjunctival implantation of G-6-3 hydrogels containing 6.4 IU insulin lispro for 1 day, 1 week, and 1 month, respectively. There was less than 0.3 μ IU insulin/mg total serum protein detected in the blood after 1 day implantation, but no lispro insulin detected in the blood after 1 week and 1 month implantation, suggesting that there was minimal to no systemic exposure of insulin after the subconjunctival implantation. The lack of systemic exposure of insulin was further supported by the data in Figure 6, showing that neither the insulin lispro loaded hydrogels nor blank hydrogels affected the blood glucose level of rats, which was approximately 100 $\text{mg} \cdot \text{dL}^{-1}$ over the 2-month period after the implantation. In previous work we reported that insulin could reach the retina after subconjunctival injection.³⁰ However, as insulin has a short half-life, daily subconjunctival injection of insulin was needed. In this work, we loaded insulin in nontoxic hydrogels and were able to achieve sustained delivery of insulin to the retina for at least 1 week after one-time subconjunctival implantation.

Effect of Insulin Released From Hydrogels on DNA Fragmentation in Diabetic Retinas After Subconjunctival Implantation

With the above data demonstrating that our nontoxic hydrogels could achieve sustained delivery of insulin to the retina for at least 1 week after subconjunctival implantation, the next step was to test if insulin released from the hydrogels would retain biological effect *in vivo*. In a previous study, we demonstrated that daily subconjunctival injection of low-dose insulin decreased DNA fragmentation in the retina of diabetic rats.³⁰ To decrease the frequency of insulin administration, we loaded insulin into G-6-3 hydrogels and implanted them for 1 week. Figure 7 shows that the control diabetic rats receiving no implantation had a 1.5 times higher retinal DNA fragmentation than the control nondiabetic rats. Subconjunctivally implanted blank hydrogels had no effect on retinal DNA fragmentation in diabetic rats 2 days and 1 week after implantation. However, after insulin lispro-loaded hydrogels were implanted in diabetic rats, the retinal DNA fragmentation

FIGURE 7. Effects of subconjunctivally implanted insulin lispro-loaded G-6-3 hydrogels on DNA fragmentation in the diabetic retina. Streptozotocin-induced diabetic rats (8 weeks) were subconjunctivally implanted with insulin lispro-loaded hydrogels or blank-hydrogels, and normal and diabetic rats without implantations were used as controls. Retinas were extracted 2 days or 1 week later and used for DNA fragmentation ELISA. “ctl” for normal rats ($n = 8$), “D” for diabetic rats ($n = 4$), “L” for insulin-loaded hydrogels ($n = 6$), and “B” for blank hydrogels ($n = 4$). $^{**}P < 0.01$ from ctl by ANOVA and Student-Newman-Keuls (SNK) post hoc test and n.s. for not significant.

in treated eyes was significantly reduced by approximately half, but the blood glucose levels were unaffected (data not shown). The results indicate that the insulin lispro-loaded hydrogels were able to rescue retinal cells from apoptosis for at least 1 week after a single subconjunctival implantation.

CONCLUSIONS

We have developed poly(NIPAAm-Dex-lactateHEMA) hydrogels that could load FITC-insulin with approximately 98% high encapsulation efficiency and sustain FITC-insulin for 5 months in vitro with tunable release kinetics. The insulin release could be slowed down by increasing the weight ratio of NIPAAm to Dex-lactateHEMA from 4:5 to 8:1 and the lactide chain length of the lactate unit in Dex-lactateHEMA, and by using DMF instead of ethanol/water (3:7, v:v) during the synthesis. The insulin release mechanism is controlled by both diffusion and degradation. Insulin lispro released from the hydrogels could increase Akt and IR tyrosine activity of ex vivo retinas. The hydrogels were not toxic to the rat retina, as evidenced by the H&E staining results showing that the hydrogels did not cause any morphological change, inflammation, or other adverse effects in the retina over a period of 2 months. Insulin released from the 1-week implanted G-6-3 hydrogels could penetrate into the rat retina in a sustained manner, as shown by confocal microscopy and RIA measurements, and rescue retinal cells from apoptosis in diabetic rats. In addition, the implanted hydrogels alone or containing low-dose insulin lispro did not change the blood glucose level of normal and diabetic rats. All these results suggest that the developed hydrogels have potential to sustain release of low-dose biologically active

insulin to the retina via subconjunctival implantation to minimize DR without the risk of hypoglycemia.

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