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The Incorporation of Extracellular Matrix Proteins in Protein Polymer Hydrogels to Improve Encapsulated Beta-cell Function

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Abstract. Biomaterial encapsulation of islets has been proposed to improve the long-term success of islet transplantation by recreating a suitable microenvironment and enhancing cell-matrix interactions that affect cellular function. Protein polymer hydrogels previously showed promise as a biocompatible scaffold by maintaining high cell viability. Here, enzymatically-crosslinked protein polymers were used to investigate the effects of varying scaffold properties and of introducing ECM proteins on the viability and function of encapsulated MIN6 β-cells. Chemical and mechanical properties of the hydrogel were modified by altering the protein concentrations while collagen IV, fibronectin, and laminin were incorporated to reestablish cell-matrix interactions lost during cell isolation. Rheology indicated all hydrogels formed quickly, resulting in robust, elastic hydrogels with Young's moduli similar to soft tissue. All hydrogels tested supported both high MIN6 β-cell viability and function and have the potential to serve as an encapsulation platform for islet cell delivery *in vivo*.

Key words: hydrogel, islet transplantation, extracellular matrix proteins

Introduction

Type 1 diabetes (T1D) is an autoimmune disease in which the insulin producing β-cells in the islets of Langerhans are selectively destroyed by auto-reactive T-cells[1-2]. Despite many advancements in exogenous insulin therapies, many patients with T1D suffer from poor glycemic control and consequently develop secondary complications such as renal disease, blindness, and neuropathy [3-4]. In addition to insulin administration, other treatment options currently available to patients with T1D include whole pancreas and/or islet transplantation. However, these options have significant drawbacks. Islet transplantation is considered primarily in patients presenting with hypoglycemia unawareness. Compared to whole pancreas transplantation, islet transplantation is relatively non-invasive and thus advantageous. Though it reduces the number of hypoglycemic episodes, islet transplantation only demonstrates sustained insulin independence in less than 10% of patients after 5 years [5].

Long-term pancreatic islet graft failure is likely due to a combination of factors. One contributing factor may be that islet cells cease interacting with their native microenvironment after isolation. Islet cell isolation is achieved through enzymatic digestion of the pancreas, liberating islets from exocrine tissue and extracellular matrix (ECM) proteins such as collagen IV, fibronectin, and laminin [6-8]. This results in a loss of the vasculature and cell-matrix connections that regulate the expression of survival genes [9-14]. A lack of islet-ECM signaling after isolation and associated cell damage has been

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correlated with reduced islet function and viability [15-19]. Additional factors that contribute to early graft failure include complications from implantation into the portal vein, such as instant blood mediated inflammatory response (IBMIR) and islet dispersion, resulting in a loss of cell-matrix connections with subsequent islet cell death [20-23].

Since the interactions between islet cells and their environment affect both their survival and function, the encapsulation of isolated islet cells in tissue-engineered biomaterial scaffolds that provide three-dimensional support may improve long-term islet engraftment and function [24,25]. Biomaterials can be designed to recreate a favorable cellular microenvironment. Some important factors to consider include material properties (such as hydrogel stiffness and elasticity) that control cell adhesion and gene expression, [26-32] as well as material porosity, which determines the diffusion of oxygen and soluble factors serving as nutrients and sensors. Additionally, the biomaterial can include key ECM components that interact with islet cell surface integrins and other trans-membrane molecules [26,27, 33-36]. These factors can all contribute to encapsulated cell viability and function [37,38].

Protein polymer hydrogels, previously investigated in our lab, may be a suitable platform for islet encapsulation and transplantation. These hydrogels are created from two different genetically-engineered, monodisperse protein polymers from tandem repetitive blocks of amino acid sequences. These polymers can be enzymatically crosslinked into modular hydrogels that vary in mechanical and chemical properties [39-42]. The genetically-engineered proteins contain either lysines or glutamines that are enzymatically crosslinked by transglutaminase to form biocompatible scaffolds for cell encapsulation [43]. Protein polymer-based hydrogels may prove superior to other scaffolds due to their biological, chemical, and physical attributes, all of which are controlled by the precise design of the amino acid building-block. Additionally, protein polymer biocompatible hydrogels have been shown to maintain a high level of cell viability and function [39,44] indicating the system may hold considerable promise for tissue engineering applications.

In the present study, a protein polymer hydrogel formulation ideal as a platform for islet cell delivery was defined by examining the physical properties of the hydrogels and assessing the viability and glucose-stimulated insulin response of encapsulated MIN6 β-cells. Secondly, the ECM proteins collagen IV, laminin, and fibronectin were incorporated into a hydrogel in different combinations to determine the effects on MIN6 β-cell function. All formulations tested showed high viability and a positive insulin response to glucose. Protein polymer hydrogels may improve islet transplantation by improving the long-term graft survival of transplanted islets.

Methods

Materials. Unless otherwise stated, all materials were purchased from Sigma Aldrich (St. Louis, MO).

Protein Polymer Synthesis and Purification. The DNA sequence and corresponding protein polymers were synthesized as previously described [40]. Two different protein sequences were expressed: **K8-30** $[GH_{10}SSGHIDDDDKHM(GKAGTGSA)_{30}G]$ and **Q6** $(GH_{10}SSGHIDDDDKHM[(GQQQLGGAGTGSA)_2(GAGQGEA)_3]_6G)$. Briefly, the DNA was constructed using the controlled cloning method [45] and inserted into modified pET-19b (Novagen, Biggstown, NJ) plasmids that were then transformed into BLR(DE3) (Novagen) cells. Cells were cultured, induced with isopropyl thiogalactoside (U.S. Biologicals, Swampscott, MA), and harvested after 4 hours by centrifugation. The protein was then lysed from the cell.

After undergoing purification with affinity chromatography using chelating sepharose fast flow nickel-charged resin (GE Healthcare, Piscataway, NJ) under denaturing conditions with competitive elution using imidazole (Fisher Scientific), the protein polymers were dialyzed and lyophilized. The molecular weight of the protein polymers was confirmed using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) on a Perseptive Biosystems

Voyager Pro DE (Applied Biosystems, Foster City, CA) or an Autoflex III Series MALDI-TOF (Bruker Daltonics, Billerica, MA) at Northwestern University's Integrated Molecular Structure Education and Research Center (IMSERC).
Endotoxins were removed from the protein polymers by phase separation. Briefly, Triton X-114 was added at 1% to protein dissolved at 10 mg/mL in endotoxin free water, and the pH of the resulting solution was adjusted to ~ 9.5. The solution was stirred for 30 min at 4ºC, placed in a 37ºC water bath for 10 min and then centrifuged at 10,000 g at 37ºC for 10 min. The supernatant containing the protein was collected and processed multiple times, with pH readjustments to ~ 9.5 after every four rounds. The solution was then placed on degassed Bio-beads SM2 Adsorbents (Bio-rad Laboratories, Hercules, CA) to remove any remaining Triton X-114, dialyzed against endotoxin free water, and lyophilized. The endotoxin levels were tested using QCL-1000 Endpoint Chromogenic LAL assay (Lonza, Walkersville, MD). An endotoxin level below 0.12 and 0.06 EU/mg of protein was required for the Q6 and K8-30 proteins, respectively.

Hydrogel formation. Hydrogels were formed through the enzymatic crosslinking of two protein polymers, K8-30 and Q6. Tissue transglutaminase (tTG) from guinea pig liver was dissolved at 0.04 units/μL in 2 mM Ethylenediaminetetraacetic acid (EDTA), 20 mM Dithiothreitol (DTT), pH 7.7. The concentrations of the K8-30 and Q6 proteins were dissolved at varying weight percents to produce the different gel formulations **(Table 1)**. The lysine-containing protein, K8-30, was dissolved in 200 mM 4-Morpholinepropanesulfonic acid (MOPS), 20 mM $CaCl_2$, pH 7.6. The glutamine-containing protein, Q6, was resuspended in 2 mM EDTA, pH 7.3. The three components were combined at a volumetric ratio of 2:3:3 of tTG:K8-30:Q6. The enzyme was added to the protein precursors and the solution was quickly mixed and pipetted onto the culture dish. All gels were incubated at 37ºC until gelation occurred. For brevity, gel formulations will be given as XQ/YK, where X represents the weight percent of Q6, and Y represents the weight percent of K8-30 in the final formulation **(Table 1)**.

Experimental Design. Initially, the material properties of protein polymer with various protein concentrations were investigated. MIN6 β-cells were then encapsulated in the protein polymers and tested for viability and glucose stimulated insulin response (IR). Next, three different ECM proteins were incorporated both individually and in combination in the protein polymer hydrogel formulation with the highest IR. The viability and IR of the MIN6 β-cells encapsulated in the hydrogel-ECM construct was then tested.

Microrheology. Particle tracking microrheology was used to determine gelation time on the microscale as previously described [46]. The particle tracking system was composed of a lab-build epifluorescent videomicroscope with a Nikon TE200 (Nikon, Melville, NY) inverted microscope using a 0.5" CCD TM-6710-CL camera. Videos were collected at 10-30 frames/second and analyzed using IDL software (ITT Visual Information Solutions, Boulder CO) and tracking code using a tau (τ) value of 1 timestep [46]. Neutral 0.2 μm fluorescent tracer particles (Molecular Probes, Eugene, OR) were added at 1:2000 dilution to combined K8-30 and Q6 that were dissolved at various weight percents. Transglutaminase was added to each solution of protein precursors, which were then deposited on a cover glass for data recording. The mean square displacement (MSD) of the particles was calculated at each time interval prior to and post enzyme addition and adjusted using a time interval of 0.0333 seconds. The reported gelation time is the time when the MSD reached 90% of the difference between the baseline without enzyme and the steady state mean after gelation occurred. Gelation time was determined for each individual measurement with the exception of the most concentrated formulation of 15Q/10K. In this case, MSD at each time point was averaged together to find a single gelation time ($n \geq 3$ for both baseline values with and without enzyme for each formulation).

Bulk Rheology. The bulk mechanical properties of the hydrogel material were determined with a Paar Physica MCR 300 Rheometer with peltier temperature control set at 37ºC and a stainless steel cone and plate (25 mm diameter, 2º angle) fixture. A

Table 1. Protein polymer hydrogel name and properties. *Hydrogel formulation by weight percent of the glutamine-protein (Q6) and the lysine-protein (K8-30). The ratio of the total number of lysine sites to glutamine sites (lys:gln) and positive to negative charge (+:-). The measured storage modulus (G') and calculated Young's modulus (E) from the frequency sweep performed at 10% strain with a Poisson's ratio of 0.5 are also represented. Representative mean square displacement (MSD) values are also shown which correlate to the sample viscosity through the generalized Stokes-Einstein relationship (Equation 1).*

Formulation Name	wt% glutamine-protein (Q6)	wt% lysine-protein (K8-30)	Ratio		G' (kPa)	E (kPa)	MSD ($\mu m^2 \times 10^{-3}$)
			lys:gln	+: -			
15Q/10K	15	10	2.1	1.0	3.1	9.2	11.0 ± 0.04
10Q/10K	10	10	3.1	1.6	1.9	5.6	22.8 ± 0.11
10Q/5K	10	5	1.6	0.8	2.4	7.3	25.4 ± 0.14
5Q/10K	5	10	6.3	3.1	2.4	7.3	27.6 ± 0.13
5Q/5K	5	5	3.1	1.6	1.9	5.6	33.5 ± 0.20

Q6 solution and a K8-30 solution of 60 µl each were mixed at the specified concentration and placed directly onto the platform with 40 µL of tTG added. A humid chamber was created around the sample using wet Kimwipes. Gel formation measurements were taken for 3 hours followed by frequency and strain sweep tests (ranging from 1-100% at an angular frequency of 10 Hz). Storage and loss modulus were measured as well in the oscillatory mode at 10% strain. The Young's modulus was calculated from the frequency sweep using the equation $E = 2G(1 + \nu)$, where E is the Young's modulus, G is the shear modulus, and ν is the Poisson's ratio. In this case, the shear modulus was assumed to be equivalent to the storage modulus (G') and the Poisson's ratio was estimated at 0.5 for a hydrogel.

MIN6* β-*cells Cell Culture. MIN6 β-cells, a mouse-derived, insulin-secreting cell line and a gift from the Kaufman lab at Northwestern University, were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose and L-glutamine (Mediatech, Inc., Manassas, VA), 75 mg/L penicillin (VWR International, West Chester, PA), 50 mg/L streptomycin (VWR International), 5 µg/L β-mercaptoethanol, and 15% fetal bovine serum (FBS) (Gemini BioProducts, West Sacramento, CA). Cells were incubated in a humidified incubator at 37ºC and 5% CO_2. The media was changed every 3 days until they reached ~80% confluence. Cells were trypsinized using 25% trypsin (Fischer Scientific).

MIN6* β-*cell Encapsulation. MIN6 β-cells were suspended in protein precursor solution and spread across a glass insert in a 35 mm microwell dish (MatTek Corporation, Ashland MA) followed by the addition of tTG. After gelation, 1 mL of media was added to the dish before transfer to the incubator (humidified at 37ºC and 5% CO_2). For viability studies, the various gel concentration formulations were first tested with 2×10^4 cells in a 25µL gel volume and then with 1×10^5 cells in a 20 µL gel volume in the presence of ECM proteins. For glucose stimulation studies with ECM proteins, 1×10^5 MIN6 β-cells were re-suspended in 7.5 µL of protein precursors and deposited on the side of the well (96-well plate). 2.5 µL of tTG was then added to make 10 µL gels. After gelation, 200 µL of media was added before transfer to the incubator (humidified at 37ºC and 5% CO_2).

Incorporation of ECM Components. Three ECM components – collagen type IV, fibronectin, and laminin (BD Biosciences, San Jose, CA) – were incorporated and tested as follows: **first** using MIN6 β-cells with each ECM component separately at 100 µg/mL per protein polymer hydrogel; **second** using MIN6 β-cells in combination with collagen IV and laminin at three different ratios: 100 µg/mL collagen IV and 50 µg/mL laminin (100C/50L); 100 µg/mL collagen IV and 100 µg/mL laminin (100C/100L); and 50 µg/mL collagen IV and 100 µg/mL laminin (50C/100L). Each ECM protein was first dissolved in 200 mM MOPS, 20 mM $CaCl_2$, pH 7.6, and then combined with the K8-30

protein at 5 wt% K8-30 and with ECM protein (K8-30-ECM). Q6 at 10 wt% and the K8-30-ECM solution were combined with tTG in a 3:3:2 volumetric ratio.

Viability. The viability of MIN6 β-cells encapsulated in protein polymer hydrogels was assessed using fluorescence staining of cell membrane integrity and intracellular esterase activity at days 1, 2, and 7. Samples were stained with 1 mL of 2 μM calcein AM (Anaspec, San Jose, CA) and 3 μM ethidium homodimer-1 (Anaspec) in phosphate-buffered saline (PBS) for 30 min at 37ºC. Viable cells fluoresced green by the reaction of calcein AM with intracellular esterases, and non-viable cells were stained red by the diffusion of ethidium homodimer-1 into cells with damaged membranes. Images were acquired (Zeiss, Germany) at either Northwestern University's Cell Imaging Facility (Chicago, IL) or Stanford University's Cell Science Imaging Facility (CSIF) (Stanford, CA). MIN6 β-cell samples (n=3), were taken from four different frames of the sample, and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Methanol-treated cells served as a negative control.

MIN6 β-cells ATP Levels In Vitro. ATP levels were assessed by combining 100 μL of media and 100 μL of Cell-titer Glo luminescence buffer (Promega, Madison, WI). Samples were shaken at 500 rpms at room temperature for 45 minutes, transferred to a white well plate (VWR International), and read on either an LB 96 V luminometer (Berthold Technologies, Oak Ridge, TN) in the High-Throughput Bioscience Center (HTBC) at Stanford University or an M5 Spectramax plate reader (Molecular Probes) at IBNAM. A standard curve of known values of ATP was used to convert the data to nanograms of ATP, which is linearly equivalent to the number of cells.

Glucose stimulated insulin response. Encapsulated MIN6 β-cells were first washed with 200 μL KREBS buffer (25 mM Hepes, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$ 0.1% BSA [pH 7.4]) without glucose exposed to two consecutive 60 minute static incubations in KREBS buffer first with low glucose (1.1mM glucose solution) and secondly with high glucose (16.7mM glucose solution). Unencapsulated MIN6 β-cells and media alone served as controls. Insulin secretion was measured using an ELISA kit (Mercodia, Winston-Salem, NC) following manufacturer's protocol. All formulations and controls were tested in duplicate. Values for MIN6 β-cells (for all conditions, n ≥ 7) were standardized to the ATP levels and normalized to the highest values in the measured plate. This method was used instead of a stimulation index due to the potential for loss of MIN6 β-cells during the assay.

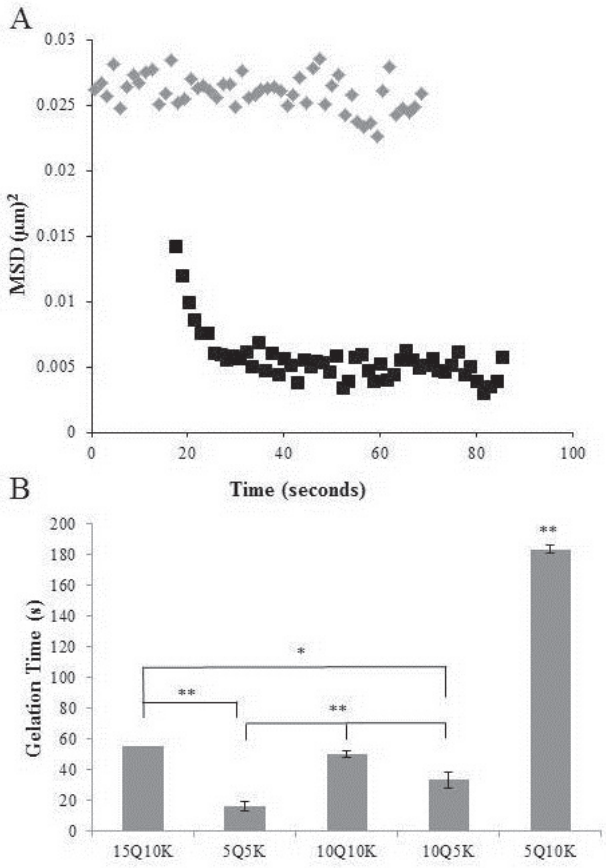

Figure 1. *Particle tracking microrheology of protein polymer hydrogels.* ***(A)*** *Example particle analysis of 10Q/5K without enzyme (◇) and with 0.04 units/μL tTG (■).* ***(B)*** *Gelation time of all formulations. A statistically significant difference with p < 0.01 is denoted with ** and p < 0.05 is denoted by *. 5Q10K is significantly greater (p < 0.01) than all other formulations.*

Statistics. Statistical analyses were performed using Excel 2007 (Microsoft Corporation, Redmond, WA) with a two-sample t-test assuming unequal variances. A p value less than 0.05 was considered significant.

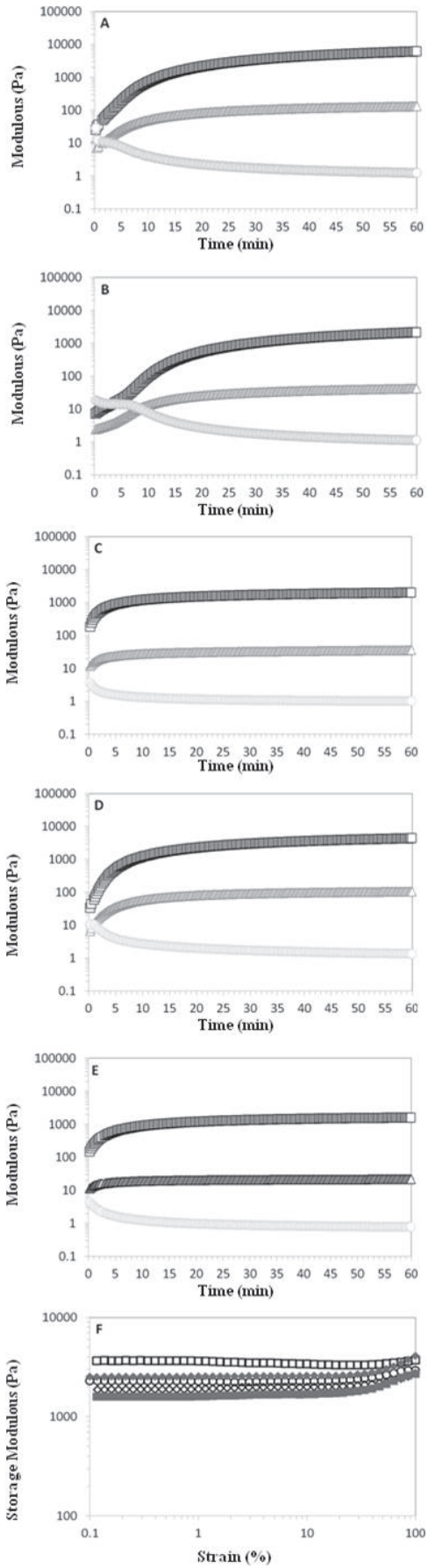

Figure 2: Hydrogel Mechanical Properties. *Moduli for formation of hydrogels with 15Q/10K **(A)**, 10Q/10K **(B)**, 10Q/5K **(C)**, 5Q/10K **(D)**, and 5Q/5K **(E)**. Each graph shows the storage modulus in Pa (G') (□), the loss modulus in Pa (G") (Δ), and the loss angle in degrees (o). Strain sweep measurements from 0-100% **(E)** of 15Q/10K (□), 10Q/10K (◊), 10Q/5K (o), 5Q/10K (♦), and 5Q/5K (■).*

Results

Microrheology. The gelation time at the microscale of different formulations of protein polymer hydrogels was determined using particle tracking microrheology. The different protein concentrations analyzed are listed in **Table 1**. A representative example of the mean square displacement (MSD) of the particles over time of 10Q/5K hydrogel shows a relatively flat curve for the baseline without enzyme and an exponential-like decrease when enzyme is added **(Figure 1A)**. The average MSD before the addition of enzyme **(Table 1)** provides a measure of the solution viscosity and indicates the impact of increasing protein precursor concentration. The MSD correlates to the sample viscosity through the generalized Stokes-Einstein relationship, listed in Equation 1, where the left-hand term is the MSD, D is the diffusion coefficient, d is the dimensionality of the particles (typically 2), a is the particle radius (here is 0.2 μm), ηo is the zero-shear viscosity, T is the temperature, τ is the lag time (time between calculated particle displacement and here is 0.0333 seconds) and the brackets indicate that it is being averaged over all starting times [47].

$$<\Delta r^{\wedge}2(\tau)>=2dD\tau=dk_B T\tau)/(3\pi\eta_o a) \quad \text{(Eq. 1)}$$

The inverse relationship between MSD and viscosity indicates that the 5Q/5K has the lowest viscosity and 15Q/10K the highest before enzyme is added **(Table 1)**.

The gelation times of each formulation are statistically different from the others ($p<0.05$) except for the 15Q/10K compared to 10Q/10K **(Figure1B)**. The 5Q/5K formulation had the lowest viscosity and the shortest gelation time (≤ 16.6 seconds) while the 5Q/10K formulation showed the longest gelation time. Lastly, the 15Q/10K formulation had a higher viscosity and gelled quickly.

Rheology. The bulk properties of the hydrogels were determined using oscillatory rheometry. Approximately 15 seconds after the addition of tTG, the storage

modulus (G') was larger than the loss modulus (G"), indicating quick formation of a gel **(Figure 2A-E)**. While the solution quickly forms a gel, the increase in the storage modulus over time indicates that crosslinking continues for an extended period of time (from 30 to 142 minutes). To determine the extent of ideal solid formation and the level of shearing tolerated by the gels, frequency and strain sweep experiments were done. During frequency sweeps, the storage modulus remained linear, indicating that the gels formed a robust elastic solid (data not shown). The Young's modulus and the storage modulus during the frequency sweep demonstrate the stiffness and viscoelasticity of the material **(Table 1)**. A small increase in the storage modulus between 20% and 40% strain was shown for all gel compositions, indicating minor damage to the gel structure **(Figure 2F)**. However, no crossover point was seen. All subsequent measurements were conducted within a linear viscoelastic region where the storage modulus was independent of the applied strain.

Viability. MIN6 β-cells were encapsulated in all gel formulations and in the 10Q/5K with individual ECM proteins at 100 μg/mL. After 1, 2 or 7 days, fluorescent microscopy showed high viability with values ranging between 91.7% and 98.9%. This suggests that the mild enzymatic gelation process and the addition of ECM proteins were well tolerated by the MIN6 β-cells.

Glucose Stimulated Insulin Response of Encapsulated MIN6 β-cells. Firstly, MIN6 β-cells were encapsulated in the different protein polymer hydrogel formulations and challenged with high glucose stimulation *in vitro*. The cells encapsulated in the 10Q/5K formulation had the highest IR (data not shown). MIN6 β-cells encapsulated in the 10Q/5K protein polymer hydrogels in the presence of collagen IV, fibronectin, or laminin were challenged with high glucose stimulation *in vitro* and compared to encapsulated cells without ECM (Gel) and to non-encapsulated cells (Cells Only) **(Figure 3A)**. After 2 and 7 days, MIN6 β-cells encapsulated with individual ECM protein had a higher insulin response (IR) per cell compared to encapsulated cells alone (Gel) and to Cells Only ($p \leq 0.01$). At 2 days, MIN6 β-cells encapsulated with either collagen IV or laminin had a higher IR (x 1.7) than cells in Gel without ECM and higher (x 3) than Cells Only. At 7 days, MIN6 β-cells encapsulated with any ECM proteins showed a similar increase in IR compared to cells in Gel (11.4 to 14.5 times higher) and Cells Only (1.9 to 2.4 times higher).

After 2 days, MIN6 β-cells, encapsulated in protein polymer hydrogels (10Q/5K) combining collagen IV and laminin, were challenged with high glucose and showed the highest IR with 100C/50L: ~1.3 times higher than for cells in Gel ($p \leq 0.05$) and ~2.4 times higher than Cells Only ($p \leq 0.01$). After 7 days, MIN6 β-cells encapsulated with the highest concentration of collagen IV (100C/50L and 100C/100L) showed similarly high IR: ~27.3 times higher than cells in Gel and ~3 times higher than Cells Only ($p \leq 0.01$).

Discussion

In developing a tissue engineering scaffold to improve the long-term islet cell engraftment, it is important to understand how the scaffold properties affect cell function and viability. In this study, a protein polymer hydrogel system previously shown to be biocompatible for tissue engineering applications was evaluated as a possible scaffold for pancreatic islet encapsulation [44]. The impact of protein concentration and the addition of ECM proteins on the hydrogel material properties and on the biological response of MIN6 β-cells was investigated.

It has previously been indicated that the survival and function of encapsulated cells is affected by both the physical and chemical properties of the hydrogel [26,48-50]. To alter the hydrogel properties (such as strength, overall charge, and chemical presentation) and determine the optimal hydrogel conditions for MIN6 β-cell survival and function, the concentration of the individual protein precursors was varied. Each molecule of Q6 contains 12 glutamines available for crosslinking, while each molecule of K8-30 presents 31 lysines (each enacting a positive charge) available for crosslinking. By changing the protein concentrations, the physical properties of the hydrogel – including the solution viscosity, overall charge and possible number of crosslinks – were altered. The overall charge of the hydrogel is important since it is well established

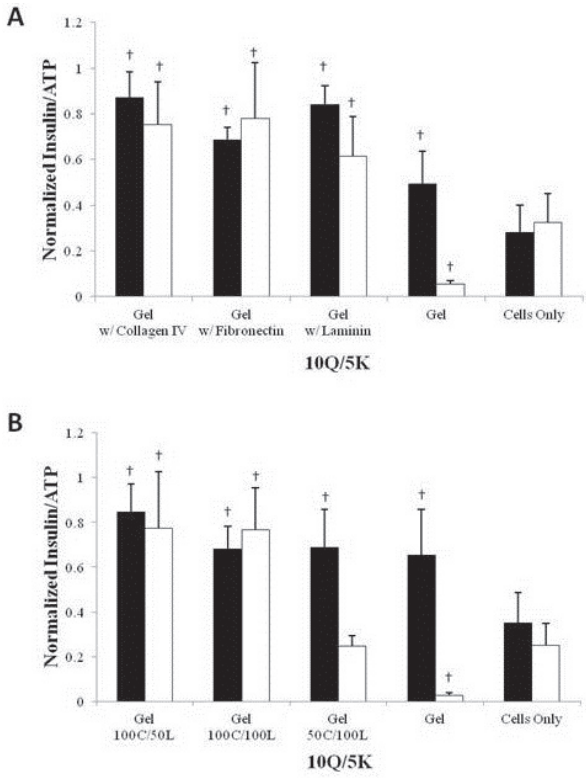

Figure 3. Beta cell function versus ECM protein concentration in a 10Q/5K protein hydrogel. *Glucose stimulated insulin response of MIN6 β-cells encapsulated with individual ECM proteins* ***(A)*** *or a combination of collagen IV and laminin proteins* ***(B)*** *after 2 (Black) or 7 (White) days in vitro. Ratios represent the normalized amount of insulin secreted in response to 16.7 mM glucose compared to the amount of ATP measured.* ***(A)*** *Individual ECM proteins were added at 100 μg/ml hydrogel.* ***(B)*** *Collagen IV (C) and laminin (L) were combined at either 50 or 100 μg/ml hydrogel. Statistics were evaluated with respect to the "Cells Only" control group at either 2 or 7 days. A statistically significant difference with a $p < 0.01$ was denoted with †. Error bars in graphs indicate means ± STDEV, $n \geq 7$.*

that excess positive charge may adversely affect the surrounding tissue and highly positive cationic proteins may be cytotoxic [51,52]. While varying the concentration of the proteins changed the chemical and physical properties of the hydrogel, all formulations resulted in quick-forming, highly elastic, robust gels that supported both high cell viability and cellular insulin response to high glucose. The 5Q/10K formulation has the highest positive charge and the 10Q/5K formulation is the only hydrogel with a net negative charge **(Table 1)**.

The material properties of the hydrogels were assessed through both microrheology, which provided a relative measure of the precursor viscosity and gelation times, and bulk rheology, which provided a measurement of the Young's Modulus and the extent to which the gels formed an ideal solid. From the microrheology studies, it appears that gelation time is a function of both the viscosity and the concentration of the lysines and glutamines. For example, the 5Q/5K formulation had the lowest viscosity and the shortest gelation time, which may be due to faster enzyme diffusion and lysine/glutamine matching. The 15Q/10K formulation on the other hand had a higher viscosity but gelled quickly since its gelation time may only be restricted by the physical proximity of lysines and glutamines. The much longer gelation time of 5Q/10K may be due to the high lysine:glutamine ratio, making it harder for the moieties to be matched and form bonds.

During bulk rheology testing, the storage modulus (G') was higher than the loss modulus (G") for all hydrogel formulations at the first recorded time point, confirming the rapid gel formation seen during microrheology. This is preferable for *in situ* hydrogels, as slow gelation can lead to inhomogeneous cell distribution [53]. However, while the material may be defined as a gel after only seconds, complete formation is not seen for several minutes as indicated by the continued increase in G' over time. Hydrogels with overall lower protein polymer concentrations and lower lysine:glutamine ratios reach steady-state faster, possibly due to the fewer number of total bonds formed. The remaining gels formed at a slower rate probably due to a high lysine:glutamine ratio, high viscosity, or a combination of both factors. The calculated Young's moduli for all formulations tested are within the physiological range of soft tissue and are similar to reported values for other hydrogels used in soft tissue engineering [27, 54-56].

ECM proteins were incorporated into the 10Q/5K hydrogel formulation, which showed the highest MIN6 β-cell insulin response. Mixing ECM proteins into the protein polymer hydrogels during gelation allows for the controlled presentation of biological factors without the concentration limitations

seen with scaffolds made from ECM proteins. Each ECM component (collagen IV, fibronectin, and laminin) was chosen due to its specific interaction with islet cells. Collagen IV is the main structural component of islet ECM and maintains islet cell attachment, migration, proliferation, differentiation, and survival [8, 18]. Fibronectin plays a role in maintaining viability and function through the inhibition of apoptosis [12,15,16]. Laminin directly interacts with collagen and cell-surface integrins known to increase glucose-stimulated insulin secretion [57-59].

The viability of MIN6 β-cells remained high after the addition of individual ECM proteins, indicating that ECM proteins do not adversely affect survival. MIN6 β-cell response glucose was greatest with the incorporation of either laminin or collagen IV into the protein polymer hydrogel. These findings coincide with previous *in vitro* and *in vivo* studies utilizing both MIN6 β-cells and murine islets [7,60]. Additionally, cells encapsulated with ECM proteins maintained high insulin response at day 7 compared to cells encapsulated without ECM proteins (Gel), thus supporting the notion that ECM proteins have a positive effect on encapsulated β cell function.

In an effort to replicate the complex native ECM microenvironment and determine if synergistic effects can be observed, collagen IV and laminin, two ECM glycoproteins known to associate with one another in vivo, were combined into the hydrogel at three different concentrations, and their effect on insulin secretion was observed [32,60]. MIN6 β-cells encapsulated in the 100C/50L and 100C/100L showed a higher insulin secretion in response to glucose at both 2 and 7 days. However, only a mild improvement in insulin secretion per cell was observed compared to collagen IV alone, suggesting the combination of ECM proteins may have minimal improvement on insulin secretion at the ECM protein concentrations investigated. While this observation is contrary to the synergistic effects observed by other groups, the unique properties of the protein polymer hydrogel may minimize the enhanced synergistic effects [61].

In vitro glucose challenges are an important tool to assess the effects of ECM proteins encapsulated islet cell function; however, *in vivo* studies can better account for host cell interactions, which may influence islet function. For example, the incorporation of ECM proteins may further improve islet function in vivo by promoting the infiltration of endothelial cells into the transplanted hydrogel, thus promoting engraftment and revascularization and preventing islet cell apoptosis due to hypoxia [62-64]. In addition, ECM proteins may also bind and store growth factors, cytokines, and other soluble signaling molecules to enhance long-term islet survival and function [65]. Lastly, by encapsulating the islets in the protein polymer hydrogel with ECM proteins, islets would not be as easily dispersed or directly exposed to toxins and immunosuppressants as they are in the portal vein circulation, the current transplantation site. Nevertheless, before these hydrogels could be used commercially, more studies are needed to address the issues of endotoxin control, optimized scale-up of the protein production, and a reliable non-animal source of tTG.

Conclusions

In this study, properties of enzymatically-crosslinked protein polymer hydrogels are investigated. Varying the concentration of the proteins changed the chemical and physical properties of the hydrogel while the addition of collagen IV, laminin, and fibronectin both individually and in combination help to better simulate the complexity of the native microenvironment. All formulations resulted in quick forming, highly elastic, robust gels that supported high viability of MIN6 β-cells with maintenance of insulin response to high glucose. However, no synergistic effects were observed with the combination of collagen IV and laminin at the concentrations tested. These *in vitro* data suggest that protein polymer hydrogels may prove to be a viable delivery platform for islet transplantation, warranting further *in vivo* studies.

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