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Macrophage embedded fibrin gels: An *in vitro* platform for assessing inflammation effects on implantable glucose sensors

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Abstract

The erroneous and unpredictable behavior of percutaneous glucose sensors just days following implantation has limited their clinical utility for diabetes management. Recent research has implicated the presence of adherent inflammatory cells as the key mitigating factor limiting sensor functionality in this period of days post-implantation. Here we present a novel *in vitro* platform to mimic the cell-embedded provisional matrix that forms adjacent to the sensor immediately after implantation for the focused investigation of the effects of early stage tissue response on sensor function. This biomimetic surrogate is formed by imbibing fibrin-based gels with physiological densities of inflammatory RAW 264.7 macrophages. When surrounding functional sensors, macrophage-embedded fibrin gels contribute to sensor signal declines that are similar in both shape and magnitude to those observed in previous whole blood and small animal studies. Signal decline in the presence of gels is both metabolically-mediated and sensitive to cell type and activation. Computational modeling of the experimental setup is also presented to validate the design by showing that the cellular glucose uptake parameters necessary to achieve such experimental declines align well with literature values. Together, these data suggest this *in vitro* provisional matrix surrogate may serve as an effective screening tool for testing the biocompatibility of future glucose sensor designs.

Keywords

Biosensors; Biocompatibility; Inflammation; Immune response; Macrophage

1. Introduction

For implantable glucose sensors to become a more reliable option for diabetes management, their useful lives must be extended beyond the period of days that is the current industry standard. All commercially available glucose sensors are of the percutaneous type, none of which have been approved for more than seven days *in vivo*. A growing body of literature is elucidating how tissue reactions to the presence of the sensor affect its function during this seven day window [1–3]. Previously, work by the authors demonstrated the negative impact that early stage inflammatory cell aggregation could have on sensor response in a whole

blood *ex vivo* system [2]. Beyond showing a diminished signal with respect to time, the previous study demonstrated through computational modeling how the presence of macrophages proximal to the sensor surface could create a “glucose depletion zone” where the interstitial concentration of glucose with respect to distance decreases near the sensor surface due to cellular glucose consumption. Additionally, it was found that the associated proteinaceous mat had virtually no effect on sensor performance [2].

The potential deleterious effects of inflammatory cell accumulation on sensor response was further demonstrated in a series of both *in vitro* and small animal *in vivo* studies [3–6]. Using a murine system, Klueh, et al. have shown that sensors implanted in both macrophage deficient and macrophage depleted mice exhibited improved performance as compared to those implanted into normal mice [6]. These studies also showed that direct injection of macrophages near the sensor insertion site generated enough of a consumptive glucose sink to dramatically decrease sensor output relative to a non-treated sensor [4].

As these previous studies show, the immune response resulting from the placement of a foreign object such as a glucose sensor in tissue is dependent upon the invasion of protein and cells from the adjacent capillary bed. It is therefore clear that an immune response to a foreign object cannot be fully recreated *in vitro* in the absence of a capillary network. However, what one can do *in vitro*, but cannot do *in vivo*, is quantitatively titrate the sensor with molecular mediators in the presence of a precise and controlled cell population. This allows one to directly observe the effect of immune cell activation and release on sensor response. In fact, much of what has been learned about the immune response to foreign surfaces, such as the rich literature of laminar flow immune cell studies, has been learned through *in vitro* testing in the absence of blood perfusion [7,8].

Our interest lies in more precisely characterizing the functional effects of the presence of immune cells in the tissue space surrounding the sensor soon after implantation, with the possibility of delineating the effects of immune cells with known regulators of the immune response. Additionally, as early stage tissue reactions to implantation occur in parallel to one another, the possibility of delineating the downstream effects of one reaction relative to another on sensor function is extremely difficult *in vivo*. For example, sensor responses during timescales at which both protein deposition and neutrophil adhesion occur are marked with erratic, declining signals that cannot be fully attributed to either process in an *in vivo* setting. A useful tool for testing early stage sensor response to immune cell aggregation and release would be a simple and characterizable *in vitro* platform to serve as a surrogate for the provisional matrix that forms around the sensor in the latter stages of inflammation and prior to the onset of tissue encapsulation. Such a surrogate would allow for the effects of early stage tissue response on sensor function to be mechanistically screened, which could inform more biocompatible designs without the immediate need for numerous *in vivo* studies.

For this purpose we adapted the fibrin gel technique of Bian et al. for the fabrication of tissue engineered 3D muscle bundles to create cell-embedded provisional matrix surrogates for testing the effect of cellular presence on implanted sensor performance [9]. This technique presents a number of features that can be used to create physiologically accurate,

biomimetic surrogates of the provisional matrix surrounding an implanted sensor. First, the construct is comprised of a fibrin gel, making it particularly applicable for the study of glucose sensors as fibrin is the primary component of the protein biofouling layer that adsorbs onto the sensor surface immediately after implantation. Additionally, it is highly tunable and robust for both cell number and type. These cell-embedded gels have been demonstrated to be able to accommodate cellular concentrations over 10^7 cells/mL for various cell types including skeletal muscle, cardiomyocytes, stem cells and epithelial cells [9–12].

In the current study fibroblasts and macrophages were embedded in 1 mL fibrin gels at a density of 2×10^6 cells/mL. Commercially available Medtronic Minimed SofSensor glucose sensors were calibrated, inserted into the gels, and the gels with sensors were immersed in a well-stirred solution of glucose-spiked cell culture medium for 24 h. To further mimic an inflammatory response, both lipopolysaccharide (LPS) and phorbol myristate acetate (PMA) served to supply macrophages with the extracellular signals that activate pro-inflammatory phenotypes [13]. LPS simulates bacterial activation of macrophages, while PMA mimics intracellular signaling functions of diacylglycerol for activation [14,15]. While each has a unique pathway to activation, they have been shown to produce pro-inflammatory cytokines through the NF- κ B pathway [16,17]. As a result of activation, both LPS and PMA treatment serve to increase glucose uptake by inflammatory cells to help them meet the large metabolic requirements necessary for immune response. Conversely, cell-embedded gels were also treated with genistein, a naturally occurring isoflavone in soybeans and tyrosine kinase inhibitor that has been shown to decrease both inflammatory cytokine production as well as extracellular glucose uptake in LPS-stimulated RAW 264.7 macrophages [18–21]. Beyond limiting glucose uptake, dietary levels of genistein have also been shown to have beneficial effects on inflammatory and metabolic disorders, though the molecular mechanisms of these results are still yet to be fully understood [20]. The sensors surrounded by inflammatory macrophage gels exhibited signal declines that recapitulated those reported previously in both whole blood and in small animal studies [2,4,6]. In this study, the extent of signal decline was proportional to the cell's metabolic activity where genistein-treated macrophages < untreated macrophages < LPS and PMA-treated macrophages. Sensors inserted in acellular fibrin gels and genistein-treated macrophage gels performed in a similar manner to bare sensors, exhibiting minimal sensor declines. Combined, these observations indicate that (1) the gels themselves cells presented no diffusive barriers to glucose transport, and (2) the sensor signal decays observed in the cell-embedded gels were metabolically-mediated.

A computational model describing glucose transport through a cell-embedded fibrin gel, combined with Michaelis–Menten kinetics to account for glucose consumption, was used to fit the observed sensor signal declines. The fitted values of K_m and V_{max} for the various cell types closely matched their published values, thus validating the experimental approach. Moving forward, we plan to develop the cell-embedded gels into a fully addressable perfused fluidic screening tool for dose-response studies of glucose sensors in a well-characterized, metabolically active environment.

2. Materials and methods

2.1. Fibrin gel fabrication

Fig. 1 outlines the experimental protocol for the construction of cell-embedded gels modified from Bian et al. [9]. The volume of these gels was specified to be 1 mL. Gels were formed by combining two solutions: (1) 200 μ L bovine plasma fibrinogen (10 mg/mL, Sigma–Aldrich, St. Louis, MO), 100 μ L Matrigel (BD Biosciences, Bedford, MA) and 480 μ L of cells suspended in media at 4.2×10^6 cells/mL and (2) 240 μ L of $2 \times$ cell media and 20 μ L bovine thrombin (50 U/mL, Sigma–Aldrich, St. Louis, MO). The $2 \times$ media and thrombin solution was then pipetted into the cell solution to make a 1 mL volume. Once diluted with the other reagents, the final cell concentration in the gel was 2×10^6 cells/mL, which is representative of the histological density of inflammatory cells surrounding an area of inflammation and wound healing as reported by Sisco et al. [22]. While other cellular densities could have been incorporated, this value was the most physiologically relevant and compared well with other studies from Klueh et al. [4,6]. Once mixed, the full solution was quickly pipetted into a cylindrical mold and allowed to crosslink for 30 min at 37 °C. Before the addition of the non-crosslinked fibrin gel solution, each cylindrical mold was pre-treated with 0.2% (wt/vol) Pluronic F-127 (Sigma–Aldrich, St. Louis, MO) to prevent adhesion of the crosslinked gel to the mold. After fabrication, the gels were removed from the mold and incubated in cell media at 37 °C for 24 h before use. This time frame is of significance as it is before the five day period where the embedded cells would begin to degrade the fibrin construct [23]. Cell viability within the gel was assessed and confirmed after fabrication and experiments via fluorescent imaging. Hoechst 33342 dye, applied at a concentration of 1 μ L/mL for 30 min at 37 °C, was used to image all cell nuclei. Calcein AM dye, applied at a concentration of 2 μ L/mL for 30 min at 37 °C, was used to image all live cells. Ethidium homodimer-1, applied at a concentration of 2 μ L/mL for 30 min at 37 °C, was used to image all dead cells. All fluorescent reagents were purchased through Invitrogen (Eugene, OR).

2.2. Cell culture

Two different cell types were incorporated into the fibrin gels for these studies. The first cell type was the RAW264.7 murine macrophage cell line (ATCC, Manassas, VA). This cell type was chosen as the model inflammatory cell in this study. The second cell type was the 3T3 murine fibroblast/pre-adipocyte cell line and was used as an alternate cell type in the subsequent sensor studies to investigate the effects of cell type on sensor response. Both cell types were cultured in low glucose (100 mg/dL) Dulbecco's Modified Eagle's Medium (DMEM, Sigma Life Sciences, St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (Sigma–Aldrich, St. Louis, MO), penicillin (50 U/mL) and streptomycin (100 μ g/mL) at 37 °C and 5% CO₂.

2.3. Functional sensor testing in fibrin gels with and without embedded cells

Similar to our previous study, commercially available Medtronic Minimed Sof-Sensors were used as the candidate sensors in this study [2]. Sensors went through a pre-treatment calibration stage to allow for sensor equilibration and to account for sensor-to-sensor variability. For this study, each sensor was incubated in low glucose (100 mg/dL) DMEM cell media for 4 h to allow a steady state, baseline current to form. Once a baseline current

had been reached for a given sensor, the sensor probe was inserted into the top of a cylindrical fibrin gel. Special care was taken to ensure that the whole sensing region was surrounded by the gel (Fig. 1).

The gel-covered sensor was then placed back in low glucose, well-stirred media and allowed to gather current readings for 24 h. As a first study, sensor readings were recorded under four conditions: (1) inserted in macrophage-embedded gels (2×10^6 cells/mL) (2) inserted in fibroblast-embedded gels (2×10^6 cells/mL), (3) inserted in acellular gels, and (4) bare sensors with no gel. The acellular gel case served as a control to examine the potential diffusive barrier that the gel itself would present to a sensor. All media in these cases were well-stirred to avoid the formation of any boundary layers at the sensor surface.

2.4. Effects of inflammatory macrophage activation on sensor signals

To more accurately recapitulate the *in vivo* post-implantation environment around the sensor, macrophages were activated to a more inflammatory phenotype before being embedded in gels. Up-regulation of inflammatory pathways and extracellular glucose uptake of macrophages was accomplished by incubation with either LPS (1 μ g/mL, Sigma–Aldrich, St. Louis, MO) for 24 h or PMA (50 nM, Sigma–Aldrich, St. Louis, MO) for 72 h to activate inflammatory pathways, thus promoting extracellular glucose uptake prior to gel fabrication. Down-regulation of inflammatory pathways and extracellular glucose uptake of macrophages was accomplished by incubation with genistein, a naturally occurring isoflavone in soybeans and tyrosine kinase inhibitor that has been shown to decrease both inflammatory cytokine production as well as extracellular glucose uptake in LPS-stimulated RAW 264.7 macrophages, at a concentration of 100 μ M for 24 h before gel fabrication [20]. The LPS, PMA and genistein dosages and incubation times were based upon previously reported protocols [18–21,24]. The testing of sensors inserted in fibrin gels embedded with LPS, PMA and genistein-treated cells followed the exact same testing protocol as described above.

2.5. Statistical analysis of experimental data

Each experimental condition was repeated in triplicate ($n = 3$). Data points were verified as being from a normal distribution using a Jarque–Bera test in MATLAB with the command “jbtest” for a value of $p < 0.05$. This test is preferred over other normality tests when comparing datasets of small sample sizes as it minimizes the occurrence of Type I errors. Data are presented as means $\pm$ standard error of the mean. A one-way repeated measures analysis of variance (ANOVA) was conducted to assess statistical significant differences amongst treatments ($p < 0.05$) in MATLAB. Bonferroni’s multiple comparison tests at a familywise error rate of 0.05 were then performed for post hoc analysis.

2.6. Validation of in vitro fibrin gel results through computational modeling

The left hand side of Fig. 2 contains a schematic representation of the numerical simulation of the cell-embedded fibrin gel experimental setup derived and implemented using MATLAB. The model was modified from our previous publications to describe an inflammatory state within the gel construct [2,25]. Briefly, transport of glucose through the environment surrounding the sensor was treated as a two compartment construct. The

compartment closest to the sensor surface (C_{gel}) represents the layer of fibrin gel and entrapped cells that surrounded the sensor during the experiment. The next compartment (C_{media}) represents the DMEM culture media that surrounded the sensor and gel. All of the modeling constants listed in Table 1 used in the simulations were obtained from literature, except the porosity of the fibrin capsule that was determined from the percent weight difference between a newly made, fully hydrated fibrin gel and fully lyophilized sample [26,27]. The porosity value of 0.975 represents a mean of three samples, and is similar to the calculated porosity of a fibrin biofouling layer ($\varepsilon = 0.91$) [2].

2.6.1. Governing equations—The governing equation describing glucose concentration in the gel layer (C_{gel}) is a non-linear, uni-dimensional diffusion-reaction equation in cylindrical coordinates through a porous medium. It has a diffusion term and a sink term to describe uptake by adherent cells (Eq. (1)).

$$\frac{\partial C_{\text{gel}}(r, t)}{\partial t} = \frac{D_{\text{gel}}}{r} \frac{\partial}{\partial r} \left(r \frac{\partial C_{\text{gel}}(r, t)}{\partial r} \right) - Q_{\text{gel}}(r, t) \quad (1)$$

Q_{gel} is defined as the rate of glucose uptake by cells in the layer and is modeled by Michaelis-Menten kinetics, where $V_{\text{max, gel}}$ is the maximum rate of consumption ($\mu\text{mol}/(\text{cell} \cdot \text{sec})$), $K_{M, \text{gel}}$ is the Michaelis-Menten constant ($\mu\text{mol}/\text{L}$), ε_{gel} is the porosity of the gel layer (unitless), and z_{cell} is the volume of a macrophage within the gel layer (L/cell) (Eq. (2)).

The Michaelis-Menten formalism applied to facilitative uptake of glucose via membrane-bound GLUT-1 and GLUT-3 transporters in RAW 264.7 macrophages is well established [18,28]. In this model, the fibrin network and cells were assumed to be well mixed within the gel layer. Therefore, they were not considered separately.

$$Q_{\text{gel}}(r, t) = \frac{V_{\text{max, gel}} C_{\text{gel}}(r, t)}{K_{M, \text{gel}} \varepsilon_{\text{gel}} + C_{\text{gel}}(r, t)} \left(\frac{1 - \varepsilon_{\text{gel}}}{z_{\text{cell}}} \right) \quad (2)$$

The governing equation for glucose concentration in the media (C_{media}) was a non-linear, uni-dimensional diffusion equation in cylindrical coordinates through a porous medium (Eq. (3)). As there are no cells in the media layer, there are no consumptive terms in the equation.

$$\frac{\partial C_{\text{media}}(r, t)}{\partial t} = \frac{D_{\text{media}}}{r} \frac{\partial}{\partial r} \left(r \frac{\partial C_{\text{media}}(r, t)}{\partial r} \right) \quad (3)$$

2.6.2. Initial and boundary conditions—For the initial condition, it was assumed that the diffusion time in the gel layer was much shorter than the time scale of simulation. Therefore, the initial condition for the system was a uniform glucose concentration (C_0) multiplied by the porosity of the gel compartment when the sensor is inserted into the media at $t = 0$ s, a plausible assumption as there was no other intrinsic source of glucose once an experiment began. The boundary condition at the sensor/layer interface was that the flux of glucose diffusion in the gel layer times the surface area (A) was equal to the rate of glucose

uptake by the sensor, Q_{sensor} . At the interface of the gel layer and media regions, it was assumed that there was no consumption or accumulation of glucose, so the concentrations normalized by the porosities and fluxes were continuous across the interface. No flux boundary conditions were assigned to the outer boundary condition at the end of the media compartment because glucose could not move into or out of the boundary at the edge of the beaker, creating an insulating boundary and making the flux at the outer boundary equal to zero. The sensor was assumed to be perfectly efficient, and the current generated by the sensor was stoichiometrically equivalent to be the amount of glucose that reached the sensor surface.

2.6.3. Nonlinear curve fitting of experimental data—Fig. 2 also outlines the general process that the computational model used to determine the best fits to the experimentally observed sensor declines. Q_{gel} is the only component with consumptive terms in the model and within that term, both the porosity of the gel and volume of a cell were assumed to be fixed in the experiment. Therefore, changes in the output of the model were dependent on only two terms, $V_{\text{max,gel}}$, the maximum rate of consumption of the cells within the gel ($\mu\text{mol}/(\text{cell}\cdot\text{sec})$) and $K_{\text{M,gel}}$, the Michaelis-Menten constant for the cells within the gel ($\mu\text{mol}/\text{L}$). The computational model described by Eqs (1)–(3) was automated to run using every possible combination of 20 different values of $V_{\text{max,gel}}$ and 20 different values of $K_{\text{M,gel}}$, all of which centered around reported values of V_{max} and K_{M} for RAW264.7 macrophages at 37 °C [18]. These centered values were designated as $V_{\text{max,o}} = 4.88 \times 10^{-11} \mu\text{mol}/(\text{cell}\cdot\text{sec})$ and $K_{\text{M,o}} = 6.13 \times 10^3 \mu\text{M}$, respectively. The output from each run of the model was the trace of the normalized glucose current as a function of time at the sensor surface, giving 400 different traces to be analyzed for a goodness of fit.

Simulations were run for a time scale of 5 h and compared to the first 5 h of the experimental data. This modification was made because the computational model assumes that macrophages will continue to consume glucose as an endless sink. In actuality analyte consumption reaches a saturation level within the cell. It was assumed that saturation was not attained within the first 5 h.

Each of the 400 modeled declines in normalized sensor current was then compared to the experimental traces of sensor decline in the presence of the following four cases: (1) untreated macrophage-embedded gel, (2) LPS-treated macrophage-embedded gel, (3) PMA-treated macrophage-embedded gel, and (4) genistein-treated macrophage-embedded gel. Non-linear coefficients of determination (R^2) were calculated to find the best fit for the four experimental conditions as one minus the residual sum of squares divided by the total sum of squares. Comparison of R^2 values was chosen for analysis at this metric quantifies how well a model describes the variation in experimental data.

2.7. Fluorescent imaging of cellular glucose uptake

To complement the data detailing the effect of cellular presence on sensor readings, fluorescent images of cellular glucose uptake were taken for each of the macrophage activation cases described above. The fluorescent glucose analog, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) (Life Technologies, Eugene,

OR), was used as the fluorescent tracer to visualize the extent of glucose uptake. Untreated, LPS-treated (1 $\mu\text{g/mL}$ for 24 h), PMA-treated (50 nM for 72 h) and genistein-treated (100 μM for 24 h) RAW264.7 macrophages were seeded separately in wells of a 24 well plate at a concentration of 10^5 cells/well in 1 mL low glucose DMEM (100 mg/dL) supplemented with 10% (v/v) fetal bovine serum (Sigma–Aldrich, St. Louis, MO), penicillin (50 U/mL) and streptomycin (100 $\mu\text{g/mL}$).

After 24 h in the wells, the cell culture media was aspirated and replaced with fresh low glucose DMEM supplemented with 100 μM 2-NBDG ($n = 3$). This value has been previously reported as the optimal concentration for imaging of the tracer [29–31]. The 2-NBDG treated cells were then incubated for 30 min at 37 °C. After incubation, the 2-NBDG-supplemented media was aspirated and each well was washed three times with sterile PBS to remove any potential background fluorescence in imaging. Fluorescent images were captured with a Nikon TE2000U microscope and NIS Elements software (Nikon) at a fixed exposure time to allow for comparisons across treatments. Images were post-processed in ImageJ.

3. Results

3.1. Cellular viability and distribution within fibrin gels

To ensure cellular viability during both the fabrication of the gel as well as the experiments with glucose sensors, live/dead staining was performed in both cases. Fig. 3 shows representative images of each case for an LPS-stimulated macrophage gel. Fig. 3a is a stained section of the cell-embedded gel immediately following fabrication, while Fig. 3b is a gel following a 24 h sensor experiment. As the post-experiment gel viability remained high ($85 \pm 5\%$, $n = 3$), we can conclude that experiments on these cell-embedded gels did not affect viability.

Beyond the issue of viability, distribution of the cells within the gel was also of importance. A desirable gel should have a uniform distribution of cells throughout the gel. This consideration ensures a uniform barrier to analyte transport to the sensor, negating any spatial heterogeneities in glucose consumption. Fig. 3c shows an image of the distribution of live cells on the surface of a cell-embedded fibrin gel 24 h after fabrication. The figure shows that cells are uniformly distributed throughout the gel, ensuring a uniform barrier to glucose transport along the length of the glucose sensor.

3.2. Sensor response in cell-embedded fibrin gels

Fig. 4 contains the average current measured from sensors over 24 h subject to four different treatments: (1) gels embedded with untreated macrophages at 2×10^6 cells/mL, (2) gels embedded with untreated fibroblasts at 2×10^6 cells/mL, (3) acellular gels or (4) gel-free bare sensors. All currents were normalized to the initial steady state baseline current for each sensor (I/I_0). Normalization controlled for sensor-to-sensor variability and allowed for multiple traces to be plotted on one graph.

The bare sensor trace never fell below 90% of the baseline value. Sensors in the acellular gels also exhibited a relatively flat response that was not significantly different from the bare

sensor trace (*) indicating that the gel itself posed no barrier to glucose transport to the sensor surface. We attributed these slight signal declines to consumption of glucose by the sensor itself. In contrast, sensors inserted in either fibroblast or macrophage-embedded gels exhibited substantial signal declines that were significantly greater than the acellular and bare sensor cases, with the macrophage decline being significantly greater than the fibroblast case ($p < 0.05$).

3.3. Effects of macrophage activation on sensor performance

The gel construct was modified to incorporate macrophages treated with either LPS or PMA to transform the cells into a proinflammatory phenotype, and genistein to block extracellular glucose uptake. The effect of these macrophage treatments on mean sensor signal is shown in Fig. 5. PMA and LPS-treated gels demonstrated the most pronounced signal decline, followed by untreated macrophage gels. Gels treated with genistein had the smallest decline in sensor signal. While not significant from one another, the PMA and LPS-treated declines were both significantly greater than the untreated case. Signal decline in the genisteintreated case was significantly lower than the untreated, LPS- and PMA-treated cases.

3.4. Characterization of fibrin gel results through computational modeling

To validate the experimental results presented above, a computational model was run to find the metabolic parameters necessary to produce the observed sensor declines. Fig. 6 presents each experimental case as well as its corresponding line of best fit for the experimental data shown in Fig. 5. Best fit fractional signal decline ($1-I/I_0$), $V_{\max}$ and K_M relative to $V_{\max,o}$ and $K_{M,o}$, and the goodness of fit (R^2) are presented in Table 2. The metabolic parameters of $V_{\max}$ ($1.15V_{\max,o}$) and K_M ($1.75K_{M,o}$) necessary to produce the best fit for the untreated macrophage trace agreed well with the values $V_{\max,o}$ and $K_{M,o}$ reported by Ahmed et al. for RAW 264.7 [18]. The goodness of fit increased and the best fit values of $V_{\max}$ all increased with the fractional signal decline. The best fit values of K_M were all larger than $K_{M,o}$, and bore no relationship to the fractional signal decline.

Classically speaking, $V_{\max}$ is the maximum rate of catalytic conversion of enzyme to substrate, and K_M has an inverse relationship to the affinity of the substrate to the enzyme. Recasting the fitted parameters relative in Table 2 relative to the untreated case (Table 3) shows more clearly that the fitted values of fractional signal decline due to cellular glucose uptake followed the same trend as the cellular glucose metabolism measured by $V_{\max}$, albeit nonlinearly. It is also interesting to note that the highest value of K_M was obtained from the fit to the genistein case where the glucose receptor was blocked.

3.5. Fluorescent imaging of cellular glucose uptake

Each case of macrophage treatment cases was dosed with the glucose analog 2-NBDG at 100 μM and imaged 30 min after treatment to visualize the differences in glucose consumption (Fig. 7). All images were captured at the optimal exposure time for the PMA-treated cells, as those had the brightest overall signal, to allow for comparison across treatments. The intensities of the intracellular glucose images in each case exhibited the same trend as that observed for the sensor signal declines where consumption by PMA-treated cells > LPS-treated cells > untreated cells > genistein-treated cells (Fig. 5). All

conditions were significant ($p < 0.05$). These results corroborate the observations from both experiments and modeling that the extent of signal decline in the cell-embedded gels arose from cellular glucose consumption.

4. Discussion

From a clinical perspective, the current utility of implantable glucose sensors for management of diabetes has been extremely limited due to their abbreviated useful lives *in vivo*. Indeed, the three to seven day window for which these devices are approved by the FDA only allows for continuous glucose monitoring as a feasible diabetes management strategy for those who require the most diligent of glucose observation. Functionally speaking, sensor behavior during this period would best be considered as anomalous and erratic, further limiting sensor efficacy even during the FDA approved time window.

Beyond being a time scale for clinical approval, the three to seven day time window coincides with a period of highly pronounced implant-associated inflammation to the newly installed sensor, punctuated by the infiltration and interrogation of the sensor surface by inflammatory neutrophils and macrophages [32]. A growing amount of evidence in recent literature has pointed to the consumptive capacity of these inflammatory cells as a potential barrier to short term sensor accuracy [2–4,6].

The design specifications for this biomimetic surrogate were chosen to maximize its physiological relevance in recapitulating early stage tissue response to an implanted sensor. First, this construct was composed of a fibrin gel. The formation of a fibrin network immediately surrounding the sensor is one of the major products of the coagulation cascade that is triggered upon insertion of the sensor into tissue, making it a primary constituent of the provisional matrix [33–37]. Secondly, the gel constructs were embedded with an inflammatory cell type at a physiologically relevant density to recreate the inflammatory cell presence around the sensor. The RAW 264.7 murine macrophage was chosen as the candidate inflammatory cell type for the gel constructs as it is a ubiquitous cell type for biocompatibility and inflammatory studies in the literature and it responds appropriately to inflammatory activation [38–42]. The density of cells within the gel was based upon *in vivo* histology data of macrophage infiltration into areas of inflammation and was also within the range of 10^5 to 10^7 cells/mL used by Klueh et al. for macrophage studies [4,22]. Lastly, this construct has been shown to not negatively impact cell viability or functionality across a wide range of cell types. The effects of interferents such as acetaminophen, urea and ascorbic acid were not considered as Medtronic Minimed sensors incorporate interference rejection membranes that negate their deleterious effects on sensor function [43].

Yet, as this construct has yet to be employed for embedding macrophages, cell viability had to be assessed before using the gels in an experiment with glucose sensors. Fig. 3a,b shows fluorescent live/dead staining for macrophages embedded in a fibrin gel. Both following the fabrication of the gel and the completion of an experiment using the gel, viability remained high. Additionally, the cells within the gel maintained a uniform distribution through the gel, as evidenced by the image in Fig. 3c. This consideration ensured that there would be an even barrier and resistance to analyte transport along the length of the active sensing region of the

sensor. Such an even layer of inflammatory cells on the surface aligns well with *in vivo* histology images where a sensor is interrogated along its whole surface by macrophages [44].

Ex vivo testing in blood and *in vivo* testing in rat subcutis have shown that the presence of macrophages in the vicinity of the sensor surface contribute to the decline of sensor signal immediately following implantation [2,4,6,44]. As a first proof of concept that cell-embedded gels would decrease sensor response when surrounding a sensor, glucose sensors were tested in four different cases (Fig. 4): (1) treatment with a macrophage-embedded gel with a cell concentration of 2×10^6 cells/mL, (2) treatment with a fibroblast-embedded gel with a cell concentration of 2×10^6 cells/mL, (3) treatment with an acellular gel or (4) treatment with no gel on the sensor surface. Fibroblasts served as an alternate cell source to ensure that any declines in sensor signal were not just in response to a bulk presence of cells, but instead were specific for cell type. The bare sensor case served as a control to examine if consumption of glucose by the sensor itself was a contributing factor to signal decrease. The acellular gel treatment served to examine if the gel itself was serving as a diffusion barrier to glucose transport to the sensor surface. Since there were no cells in these gels, the protocol was modified such that 480 μ L of cell media was added to the gel formulation instead of a cellular solution of media.

The signal of the bare sensor never dropped below 90% of its pre-treatment baseline output signal over 24 h, which was negligible in comparison to the drops observed by sensors surrounded by cell-embedded gels. When inserted into acellular gels, sensors maintained signals that were redolent of a bare sensor in media. Indeed, the acellular gel case and the bare sensor case were not statistically significant from one another ($p > 0.05$), making it the only case comparison in Fig. 4 that was not statistically significantly different. From these data, it can be concluded that the gel itself does not provide any diffusive barrier to glucose transport to the sensor. This finding was not surprising given that greater than 97% of the gel is void space, which is comparable to the 91% void space calculated for a fibrin biofouling layer [2]. Additionally, the results of this case show that the gel does not have a deleterious impact on the enzyme activity of the sensor.

The presence of cell-embedded gels did have a substantial impact on sensor signal in both the fibroblast and macrophage-embedded cases in Fig. 4, causing declines to 50% and 30% of their respective original signals, respectively. Both cases were statistically significantly different from each other as well as the bare sensor and acellular cases ($p < 0.05$). The smaller signal decline observed for fibroblast-embedded gels vs. macrophage-embedded gels was anticipated as pre-adipocyte fibroblasts have a lower reported $V_{\max}$ (1.67×10^{-12} μ mol/(cell-sec)) than macrophages (4.88×10^{-11} μ mol/(cell-sec)) on a per-cell basis at 37 $^{\circ}$ C, thereby showing that the gel construct was also sensitive to changes in cell type [18,45,46].

After the initial verification that cells could reliably maintain viability and produce a drop in signal that is sensitive to cell type, a more extensive study was conducted using activated macrophages. Four gel cases were employed in this study: (1) gels embedded with LPS-treated macrophages, (2) gels embedded with PMA-treated macrophages, (3) gels embedded

with untreated macrophages and (4) gels embedded with genistein-treated macrophages (Fig. 5). The purposes of this study were twofold. First, these treatments served as verification that the observed decrease in signal was metabolically regulated instead of a case where the presence of cells in the void space of the gel retarded glucose diffusion to the sensor via steric exclusion. Secondly, activated macrophages present a more physiologically correct model for macrophage phenotype and behavior within the site of implantation. Therefore, to provide a more complete *in vitro* model of sensor behavior, the effect of cell activation had to be considered.

As the negative control treatment, the genistein-treated case produced modest declines in signal, dropping only ~20% for the length of the experiment. This result is expected as genistein has been shown to be a competitive inhibitor of glucose uptake, decreasing the efficacy of two predominant facilitative glucose transporters, GLUT-1 and GLUT-3 to extracellular glucose across cell types [18,47,48]. Additionally, this case displays that uptake of the analyte is mediated through facilitative diffusion and not passive diffusion through the cell membrane.

Macrophage activation through both LPS and PMA treatment was shown to have an effect on sensor signal relative to both the untreated macrophage case and the genistein-treated macrophage case. However, these treated cases did not differ significantly from one another. Given the considerable metabolic demands that activated macrophages face during the foreign body response in the way of attempted phagocytosis, respiratory burst, cytokine production and cellular recruitment, it is plausible that these treatments would increase the uptake of glucose. Treatment with each activator has been shown to increase cell surface transporter affinities for extracellular glucose in macrophages, effectively raising the maximal rate of glucose uptake [49–51].

The trends of macrophage-mediated sensor signal decline seen in Fig. 5 were confirmed through the uptake of a fluorescent analog of glucose, 2-NBDG, in each of the four cases detailed above (Fig. 7). 2-NBDG serves as an ideal analog for glucose as its uptake has been shown to be facilitated through the same major transporters as glucose, thus providing an accurate basis for comparison [30]. As it is metabolized to a phosphorylated fluorescent derivative and decomposed into a non-fluorescent constituent, the optimal staining time is a dynamic equilibrium between uptake and decomposition [52]. For this cell type, the optimal staining time was determined to be 30 min for a 2-NBDG dose of 100 μ M. The short time of incubation also avoided the possibility of the fluorescent signal quenching with increased exposure to the tracer. At this time, the effects of activation on uptake are clear, as the intensity in the PMA-stimulated case is brighter than the genistein-stimulated traces just by inspection in the inset of Fig. 7. The PMA-stimulated traces are brightest on a per-cell basis, which is consistent with the trends seen in the first 30 min of Fig. 7. Predictably, the fluorescent signals in the genistein-stimulated case are faint and barely perceptible over the background, which is expected given that genistein is known to decrease GLUT transporter affinity to the analyte.

To add context to the results of the *in vitro* cell-embedded gel design, the unstimulated and LPS-stimulated macrophage cases can be compared to the maximal and minimal *ex vivo*

results for sensor decline presented in our previous study [2]. Both traces lie within the envelope outlined by the maximal and minimal *ex vivo* declines, demonstrating that the effects seen with the *in vitro* gel system are adequate representations of what would occur in a more realistic, clinical setting. Moreover, the magnitudes of decline seen in these cases are very similar to those reported in both *in vitro* and *in vivo* studies by Klueh et al. for comparable (~24 h) time frames [4]. During *in vivo* studies where 2.5×10^6 macrophages were injected at the site of implantation in a mouse, the group witnessed sensor declines to between approximately 20% and 35% of the pretreatment signals over 24 h. Similarly during *in vitro* studies where macrophages were injected directly on the sensor surface, signals declined in the same fashion over the period of 24 h. Favorable comparisons between the results of Fig. 5 and previously published reports of sensor decline in the presence of inflammatory cells demonstrate the utility that this construct could have as an *in vitro* screening tool for sensor biocompatibility.

Mechanistic characterization of the fibrin gel design was accomplished using a computational model of the experimental setup to quantify the metabolic parameters necessary to produce the experimental declines seen in Fig. 5. The major consideration in performing such a validation was due to the behavior of the cells once cultured in a 3D environment. Metabolic characteristics for macrophages, even RAW 264.7 macrophages in particular, are well characterized for a number of different conditions, including variations in temperature and treatment type [49–51,53]. However, all of these studies have been based on macrophages cultured in a 2D monolayer on a substrate like tissue culture polystyrene that has been optimized for cell culture. When cell culture is moved from a 2D monolayer to a 3D construct like the fibrin gel, a number of complex factors – from the physical forces on the cells themselves to the interaction between the cells and the surrounding micro-environment – can alter cell phenotype and potentially negatively impact their functional capabilities relative to a 2D monolayer [54,55]. Therefore, this model was implemented both to test whether the $V_{\max}$ and K_M values necessary to recreate the results from Fig. 5 were reasonable relative to published literature values and to determine whether changes in trends across treatments aligned with previously reported results.

Best fits were determined through a screening process where modeled results were compared to experimental traces through comparison of R^2 values (Tables 2 and 3). The best fits described the variations in the observed experimental data well, with R^2 values ranging from 0.715 in the genistein-stimulated macrophage case to 0.989 in the PMA-stimulated macrophage case. The best fit for unstimulated macrophage case was found to have a $V_{\max}$ of $1.15V_{\max,o}$ and a K_M of $1.75K_{M,o}$. These results agree well with the benchmark values of each reported by Ahmed et al. and demonstrate that encapsulation within the 3D fibrin gel substrate does not adversely affect cell function with respect to glucose uptake [18].

Likewise, best fits of traces from activation of macrophages with LPS and PMA resulted in increased consumptive parameters in each case. The best fit of the PMA-stimulated macrophage case had a $V_{\max}$ of $6.8V_{\max,o}$ and a K_M of $3.7K_{M,o}$. While there is not a direct comparison of fold increase in $V_{\max}$ and K_M from PMA-stimulated RAW 264.7 macrophages, Kiyotaki, et al. reported a two-fold increase in the $V_{\max}$ of J774 murine macrophages after only 3 min of PMA incubation at a concentration of 6 $\mu\text{g/mL}$ when

compared to unstimulated macrophages [56]. Ahmed et al. reported a 4 fold increase in glucose transporter affinity to glucose when RAW264.7 macrophages were cultured with PMA for 30 min [18]. The best fit of the LPS-stimulated macrophage case had a $V_{\max}$ of $2.2V_{\max,0}$ and a K_M of $2.5K_{M,0}$. These results agree well with the findings of both Gamelli et al. and Fukuzumi et al., who observed two to three-fold increases in maximal glucose consumption rate in macrophages stimulated with LPS [49,50]. Overall, the increases in $V_{\max}$ calculated from the stimulated cases make sense from a physical perspective. With an increased demand for the analyte during inflammatory stimulation, cells would be expected to increase their rate of consumption to meet such metabolic demands. While it should be noted that the model was more sensitive to changes in $V_{\max}$ than K_M , it was nonetheless surprising that stimulation was met with increases in K_M . Increases in K_M suggest that the transporters are becoming less effective in their analyte uptake. For cases of inflammatory stimulation, one would think that K_M would decrease, as that is representative of an increase in transporter affinity to the analyte. Despite these decreases in the affinity with which the cells are taking up the analyte, their rate of maximal consumption on a per-cell basis is increased, which over time will translate to higher consumption in the provisional matrix. Taken in concert, these stimulated case model fits further demonstrate the utility of the fibrin gel construct by showing that stimulated cells in a 3D environment have similar increased glucose uptake profiles to stimulated cells in a 2D environment.

This proof of concept study demonstrates the feasibility of using a fibrin-based, cell-embedded *in vitro* provisional matrix surrogate to study early stage glucose sensor biocompatibility. Moving forward, this platform could also be extended to investigate the effects of other possible mitigating *in vivo* factors on sensor function. An obvious extension of this system could investigate how these tissue responses affect oxygen transport. As oxygen is a necessary input for glucose oxidase-based glucose sensors to produce a signal, a thorough examination of a depleted sensing environment should also include the interaction between tissue and oxygen proximal to the sensor. Inflammatory cells have elevated affinity for oxygen to facilitate anti-microbial defense mechanisms like respiratory burst. To further complicate matters, the tissue wounded from implantation is shown to have decreased oxygen tension relative to untreated tissue [57–60]. Such an increased affinity for oxygen is exacerbated by the decreased amount of oxygen present in wounded tissue. Gough et al. have demonstrated that oxygen permeability within tissue surrounding an implant decreases over time before arriving at an asymptotic value, with the steepest drop coming in the first week [61,62]. Additionally, the concentration of unbound oxygen in tissue is much lower than that of glucose, meaning that oxygen limits the glucose oxidase reaction in the body [63,64]. This situation of both increased demand and limited supply of oxygen could negatively impact sensor function where the sensor cannot produce hydrogen peroxide despite the presence of glucose. In effect, the sensor would become more sensitive to oxygen than glucose. By incorporating the fibrin gel setup with varying concentrations of environmental oxygen, one could investigate the possible synergistic effects of limited glucose and oxygen supply on sensor function.

5. Conclusions

In this study, a biomimetic surrogate of early stage tissue reaction to implantation was fabricated and used as a tool to assess glucose sensor performance in the presence of pro-inflammatory macrophages proximal to the sensor surface. When inserted into macrophage-embedded fibrin gels, sensors produced declines over 24 h that were comparable to previous *ex vivo* and *in vivo* studies with glucose uptake parameters that were similar to those reported in the literature. Additionally, the versatility of the design was demonstrated as gel constructs were shown to be responsive to cellular stimulation, as sensors inserted into both LPS and PMA-stimulated macrophage gels produced significant drops in signal relative to the unstimulated macrophage case. As the causal link between tissue reaction and sensor function continues to be clarified, this *in vitro* fibrin gel system presents an exciting and promising platform for assessing early stage sensor function without the need for immediate *in vivo* studies.

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Fig. 1.
Outline of fabrication and testing of cell-embedded fibrin gels.

Fig 2.

Computational flowchart to determine which glucose uptake parameters best fit experimental data of glucose sensor signal decline. The flowchart is generalized for 4 values of V_{max} and 2 values of K_M to illustrate the process.

Fig. 3.

(a) Live/dead assay of a cell-embedded fibrin gel 24 h after fabrication. Scale bar 200 μm. (b) Live/dead assay of a cell-embedded fibrin gel immediately following a 24 h experiment when surrounding a glucose sensor. Scale bar 100 μm. Live cells are stained green, dead cells are stained red and all nuclei are stained blue. (c) Live cell Calcein AM staining of distribution of cells on the surface of a cell-embedded fibrin gel 24 h after fabrication. Scale bar 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig 4.

The effect of the presence of cell-embedded fibrin gels on glucose sensor performance. Data represented as mean normalized glucose sensor currents $\pm$ SEM ($n = 3$). * represents treatments that were not statistically significant from one another ($p > 0.05$).

Fig. 5.

The effect of macrophage stimulation in fibrin gels on glucose sensor performance. Data represented as mean normalized glucose sensor currents $\pm$ SEM ($n = 3$). * represents treatments that were not statistically significant from one another ($p > 0.05$).

Fig. 6.

Computational best fits of cases where a PMA-treated macrophage gel (green), an LPS-treated macrophage gel (red), an unstimulated macrophage gel (blue) and a genistein-treated macrophage gel (black) surround a sensor. The best fit for each case is plotted with dotted lines in the same color as the corresponding experimental case. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Mean fluorescent intensity (arbitrary units) of cellular uptake of 100 μ M 2-NBDG uptake 30 min after administration for PMA-treated, LPS-treated, unstimulated, and genistein-treated RAW 264.7 macrophages (mean $\pm$ SEM, $n = 3$). All cases are statistically significant different from one another ($p < 0.05$). Inset: Representative fluorescent images of PMA-treated and genistein-treated cells to show qualitative differences in fluorescence. Scale bar: 100 μ m.

Table 1

List of model parameters for computational model of glucose transport.

Model parameters	Parameter values		
	Gel layer	Media	Citation
Diffusion coefficient (D) (cm ² /sec)	6.8×10^{-6}	6.8×10^{-6}	[26]
Porosity (ϵ) (unitless)	0.975	–	**
Layer thickness (L) (cm)	0.43	–	**
Michaelis Menten constant (K_M) (μ M)	6.13×10^3		[18]
Maximal rate of cellular glucose uptake ($V_{\max}$) (μ mol/(cell-sec))	4.88×10^{-11}		[18]
Cell volume (z_{cell}) (L/cell)	2.1×10^{-12}	–	[27]
Glucose sensor radius (a) (cm)	0.07	–	[25]
Sensor surface area (A) (cm ²)	0.0058	–	[25]

Table 2

Values of best fits for experimental declines in sensor signal.

Treatment	Signal decline ($1-I/I_0$) at 5h	R ² of Fit	V _{Max} relative to V _{Max,o} *	K _M relative to K _{M,o} *
PMA	0.78	0.989	6.8	3.7
LPS	0.58	0.943	2.2	2.5
Untreated	0.38	0.845	1.15	1.75
Genistein	0.22	0.715	1.05	3.8

* Michaelis-Menten values for glucose metabolism in RAW 264.7 macrophages reported by Ahmed et al.: $V_{\max,o} = 4.88 \times 10^{-11}$ $\mu\text{mol}/(\text{cell}\cdot\text{sec})$ and $K_{M,o} = 6.13 \times 10^3$ μM [18].

Values in green are meant to show a fold increase relative to $V_{\max,o}$ and $K_{M,o}$, whereas values in red are meant to show a fold decrease relative to $V_{\max,o}$ and $K_{M,o}$.

Table 3

Best fit model values for all macrophage treatment cases relative to the best fit values for the untreated case.

Treatment	Fold change $1-I/I_0$ relative to untreated	V_{Max} Fold change relative to untreated	K_M Fold change relative untreated
PMA	2.05	5.91	2.11
LPS	1.52	1.91	1.42
Untreated	1.00	1.00	1.00
Genistein	0.58	0.91	2.17

Values in green are meant to show a fold increase relative to the untreated case, whereas values in red are meant to show a fold decrease relative to the untreated case.