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Functionalized PEG hydrogels through reactive dip-coating for the formation of immunoactive barriers

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Abstract

Influencing the host immune system via implantable cell-delivery devices has the potential to reduce inflammation at the transplant site and increase the likelihood of tissue acceptance. Towards this goal, an enzymatically-initiated, dip-coating technique is adapted to fabricate conformal hydrogel layers and to create immunoactive polymer coatings on cell-laden poly(ethylene glycol) (PEG) hydrogels. Glucose oxidase (GOx)-initiated dip coatings enable the rapid formation of uniform, PEG-based coatings on the surfaces of PEG hydrogels, with thicknesses up to 500 μm where the thickness is proportional to the reaction time. Biofunctional coatings were fabricated by thiolating biomolecules that were subsequently covalently incorporated into the coating layer via thiol-acrylate copolymerization. The presence of these proteins was verified via fluorescent confocal microscopy and a modified ELISA, which indicated IgG concentrations as high as 13 ± 1 ng / coated cm^2 were achievable. Anti-Fas antibody, known to induce T cell apoptosis, was incorporated into coatings, with or without the addition of ICAM-1 to promote T cell interaction with the functionalized coating. Jurkat T cells were seeded atop functionalized coatings and the induction of apoptosis was measured as an indicator of coating bioactivity. After 48 hours of interaction with the functionalized coatings, $61 \pm 9\%$ of all cells were either apoptotic or dead, compared to only $18 \pm 5\%$ of T cells on non-functionalized coatings. Finally, the cytocompatibility of the surface-initiated GOx coating process was confirmed by modifying gels with either encapsulated β -cells or 3T3 fibroblasts within a gel that contained a PEG methacrylate coating.

Keywords

Apoptosis; Cell Encapsulation; Immunomodulation; Lymphocyte; Surface Modification

1. INTRODUCTION

Strategies to encapsulate donor tissues within biomaterials prior to transplantation have been extensively investigated to reduce immune-mediated damage and promote cell survival. Encapsulated tissue transplants hold the potential to cure numerous diseases resulting from

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the loss of endocrine, cardiovascular, and neurological tissues [1]. One of the most widely studied diseases treatable with transplanted tissues is type I diabetes mellitus (T1DM). T1DM results from the autoimmune destruction of insulin-producing β -cells, located within the pancreatic islet of Langerhans cell clusters. The number of successful pancreatic tissue transplants continues to increase [2, 3] and transplant recipients experience fewer complications resulting from diabetes, compared to patients treated with daily insulin injections alone [4]. Despite continued improvements in transplantation regimens, most transplant recipients require systemic immune suppression to prevent graft rejection [2], which can lead to undesirable side effects including an increased risk of infection. Researchers have hypothesized that cell encapsulation within semi-permeable barriers prior to implantation may reduce or eliminate the requirement for systemic immunosuppression by reducing contact between donor tissue and the host immune system [1, 5]. Thus, strategies to encapsulate cells within natural and synthetic biomaterials have been extensively investigated [6]. Unfortunately, in the absence of systemic immunosuppression, encapsulated cell transplants are still likely to fail, in part because small cytokines and reactive oxygen species (ROS) are secreted by nearby immune cells, and these species remain able to diffuse into the capsule [1, 5] where they damage or destroy encapsulated cells [5, 7, 8].

To improve semi-permeable encapsulation barriers, researchers in our laboratory, as well as others, have investigated methods to functionalize biomaterials to actively modulate the local immune environment. For example, antibodies and peptides have been incorporated within capsules to bind and sequester damaging cytokines [9, 10]. Enzymes may be incorporated to breakdown ROS, like superoxide, and promote cell survival [11–14]. Further, materials have been tuned to elute anti-inflammatory molecules for localized immune suppression [14–17]. Each of these strategies has shown potential to provide protection for encapsulated cells, but no method of immune modulation has been sufficient to allow long-term transplant survival in the absence of systemic immunosuppression. Towards reducing the local concentration of activated immune cells responding to a transplant, we have recently reported a polymeric coating that induces apoptosis in T cells [18, 19], the adaptive immune cells implicated in mediating the long-term rejection of encapsulated cell transplants [20]. This coating utilized immobilized anti-fas antibody, known to induce T cell apoptosis upon engagement of the T cell fas receptor [21], and an adhesion ligand, ICAM-1 [22]. A dithiocarbamate (DTC) photoinitiator was incorporated on a hard, plastic substrate to initiate poly(ethylene glycol) (PEG) polymer chain formation with pendant anti-fas and ICAM-1 [23]. Using this chemistry, polymeric surfaces were coated with precise thicknesses and a high surface density of available biomolecules. These functionalized coatings induced T cell apoptosis and demonstrated the potential of functionalized PEG-based polymer coatings to interact with local immune cells [18]. While this previously-investigated photoinitiator-based photopolymerization enabled the formation of bioactive polymer chains, coating of cell-laden biomaterials was hindered by limited chemical reactivity in aqueous conditions, as well as technical difficulties in forming a conformal coating on an arbitrarily shaped 3D hydrogel. Thus, an alternate strategy is investigated herein.

Many biomaterials promote encapsulated cell viability and function [6], including PEG diacrylate (PEGDA) hydrogels [24–26]. PEG hydrogels have several characteristics which make them desirable for cell encapsulation and transplantation, including a high water content which can mimic native tissue, the potential for simple chemical modifications to incorporate biomolecules, and limited immunogenicity *in vivo*. Thus, PEG hydrogels were selected as the model encapsulation material for this study. To signal apoptosis in a local population of T cells, simple approaches like the co-encapsulation of anti-fas antibody within the cell-laden hydrogel are inadequate because fas signaling is damaging to many cell

types, including β -cells, and anti-fas must be physically separated from encapsulated cells [27]. Therefore, it is necessary to selectively modify the exterior of the cell-laden hydrogel with anti-fas to prevent contact with the encapsulated β -cells. Several strategies have been previously employed to functionalize the surfaces of PEG hydrogels. For example, Weber *et al.* fabricated layer-by-layer (LBL) hydrogels by immersing islet-laden PEG hydrogels into PEGDA precursor solution and photopolymerizing [28]. Likewise, Hahn *et al.* adapted traditional photolithography to pattern 3D structures and biomolecules onto the surfaces of PEG hydrogels [29, 30]. Microcontact printing, or soft lithography, has also been employed to stamp biomolecular patterns onto hydrogel surfaces [31]. While each of these modification methods provide specific advantages, it remains difficult to apply these techniques to fabricate uniform conformal coatings on 3D, cell-laden hydrogels. Though traditional photolithography allows patterning on 2D surfaces, light attenuation prevents the simultaneous formation of uniform coatings on all facets of a 3D hydrogel. Likewise, soft lithography allows for versatile transfer of 2D patterns, but functionalizing multiple faces of a 3D object would be quite complex with this method and edges would inevitably be different in their coating as compared to the surfaces.

Recently, Johnson *et al.* introduced an innovative strategy for fabricating uniform and sequential layers of hydrogel coatings [32]. They demonstrated the formation of polymer coatings via radicals generated by the reaction of glucose with glucose oxidase (GOx) at a hydrogel surface [32]. When glucose diffuses out of a pre-swollen gel and reacts with GOx, hydrogen peroxide (H_2O_2) is generated and further reacts with Fe^{2+} to form a hydroxyl radical ($HO\bullet$) capable of initiating polymerization [33]. As Johnson *et al.* demonstrated, when a PEG hydrogel was swollen in a glucose solution and then dipped into a glucose-free pre-polymer solution (PEGDA, GOx and Fe^{2+}), glucose diffuses out of all faces of the hydrogel, reacts with GOx and initiates polymerization at the surface, as summarized in Figs. 1A–C [32]. This reaction results in the rapid formation of polymer coatings with tunable thicknesses ranging from approx 100 to 800 μm , with the thickness proportional to the reaction time and dependent on the glucose concentration [32]. Further, this chemistry is possible in the presence of encapsulated cells [33]. Thus, the simplicity, cytocompatibility and ability to fabricate coatings of precisely controlled thicknesses on 3D hydrogels makes GOx-mediated dip-coating desirable for functionalizing β -cell-laden hydrogels for immune cell signaling. In our present report, we modify the previously-reported GOx-initiated polymer coating strategy to modify cell-laden hydrogels with conformal coatings functionalized with anti-fas antibody and ICAM-1 and examine the ability of these coatings to induce T cell apoptosis.

2. MATERIALS & METHODS

2.1 Materials

Poly(ethylene glycol), poly(ethylene glycol) monomethacrylate, triethyl-amine, acryloyl chloride, glucose oxidase enzyme, Fe^{2+} , catalase, bovine serum albumin, EDTA, and D-glucose were obtained from Sigma-Aldrich, St. Louis MO. All cell culture media, trypsin EDTA, fetal bovine serum, penicillin/streptomycin, fungizone, and live/dead staining assay were obtained from Invitrogen, Carlsbad, CA. Anti-fas antibody (DX2 clone) and ICAM-1/Fc chimera were obtained from R&D Systems, Minneapolis, MN. All other antibodies were obtained from Jackson ImmunoResearch, West Grove, PA. Traut's reagent, Zeba Spin filtration columns, Ellman's reagent, fluoroldehyde reagent solution, and TMB ELISA substrate were each obtained from Thermo Fisher Scientific, Rockford, IL. 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959) was obtained from Ciba Specialty Chemicals, Basel, Switzerland. Cell Titer Glo viability assay was obtained from Promega, Madison, WI. All compounds were used as received without additional purification steps.

2.2 Cell culture

All cells were cultured at 37°C, in 5% CO₂ and humid conditions. Min6 β-cells and Jurkat T cells were maintained in RPMI 1640 media supplemented with fetal bovine serum (FBS, 10%), penicillin/streptomycin (1%) and fungizone (0.5 µg/ml). Jurkat T cells were passaged every other day and seeded at 2.5×10⁵ cell/ml. Min6 β-cells were passaged once per week by treatment with 0.5 % trypsin EDTA (10 ml, 5 min) and seeded at 1×10⁶ cells/ml. Fresh Min6 media was exchanged every other day. 3T3 fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (FBS, 10%), penicillin/streptomycin (1%) and fungizone (0.5 µg/ml). 3T3s were passaged every 4 days by treatment with 0.5 % trypsin EDTA (10 ml, 5 min) and seeded at 1×10⁵ cell/ml.

2.3 Synthesis of poly(ethylene glycol) diacrylate (PEGDA) and formation of hydrogels

The synthesis of PEGDA has been described elsewhere [9, 24] and will be summarized herein. Briefly, hydroxyl-terminated PEG (10 kDa) was dissolved in anhydrous toluene at 60°C and then allowed to cool to room temperature (RT). A 4-fold molar excess (per hydroxyl group) of triethylamine (TEA) and acryloyl chloride was added and the reaction was allowed to proceed overnight at RT. The following day, PEGDA was filtered through neutral alumina to remove TEA and then placed on the rotavap to remove excess toluene. Finally, PEGDA was precipitated in diethyl ether and desiccated to dryness overnight. A PEGDA (10 wt%, 10 kDa) hydrogel was used as the underlying substrate for all coating studies. Hydrogels were formed from pre-polymer solution containing PEGDA (10 kDa, 10 wt%) and 0.5 wt% 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959, as a photoinitiator) in phosphate buffered saline (PBS, pH = 7.4). 30 µl pre-polymer was added to the tip of a 1 ml syringe and polymerized for 9 minutes under ultraviolet light (centered at 365 nm, 6 mW/cm²). Following polymerization, solid hydrogel disks were ejected from the syringe tips into >3 ml cell culture media (RPMI 1640 supplemented with 10 % fetal bovine serum (FBS), 100 mg/ml penicillin/streptomycin, and 0.5 mg/ml Fungizone) and incubated overnight at 37°C, with 5% CO₂ and humid conditions.

2.4 Thiolation of IgG, anti-Fas, & ICAM-1

To covalently incorporate proteins within polymer coatings, proteins were modified via the addition of thiol groups. As previously demonstrated in our laboratory, proteins and peptides with free thiol groups may be photopolymerized via the radical-mediated thiol-acrylate polymerization [34]. Free thiol groups were added to IgGs, anti-Fas (IgG, DX2 clone), and ICAM-1 (1 mg/ml) by reacting with Traut's reagent (5 mol Traut's reagent / 1 mol protein), in PBS containing 5 mM EDTA for 1 hr with mixing. Unreacted Traut's reagent was removed via filtration through Zeba™ Spin Desalting Columns (7K MWCO). Protein solutions were diluted to a final concentration of 1 mg/ml in PBS containing bovine serum albumin (BSA, 2 mg/ml) and EDTA (5 mM) and frozen at -80°C. Thiolated proteins were thawed immediately prior to polymerization into polymer coatings.

2.5 Formation of GOx-mediated Polymer coatings

After swelling overnight in cell culture media, a polymer coating was formed on hydrogels using the reaction of glucose oxidase with glucose, in the presence of Fe²⁺, to generate radicals capable of initiating polymerization. Hydrogels swollen overnight (>12 hrs) in cell culture media (containing 11.1 mM glucose) were dipped in pre-polymer solutions containing either PEG acrylate (acryl-PEG, 25 wt%, M_n=400 Da) or PEG methacrylate (methacryl-PEG, 25 wt%, M_n=525 Da), with Fe²⁺ (4 mM) and GOx (0 to 50 µM) in phosphate buffered saline (PBS, pH=7.4) for subsequent coating formation. Methacrylated Rhodamine (methacryl-Rhod, 300 µM) was included in some coatings for visualization purposes, but did not significantly affect the polymerization or polymer film growth rate.

Thiolated IgGs (from goat, blank and FitC-conjugated) and anti-Fas were incorporated into coatings at 250 $\mu\text{g/ml}$. Thiolated ICAM-1 was incorporated at 100 $\mu\text{g/ml}$. Gels were dipped in 50 μl pre-polymer in individual wells of a 96-well plate for 0 to 60 s to study varying times of coating formation. After dipping, coated hydrogels were removed and immersed in either cell culture media or PBS for further study.

2.6 Characterization of coatings

The thickness of the polymer coatings as a function of dipping time was measured via laser-scanning confocal microscopy (LSM 500 microscope, Zeiss). Methacryl-Rhod was co-polymerized into PEG coatings for visualization and a series of images were taken at 10 μm increments throughout the full thickness of coated hydrogels. Images were reconstructed into a 3D model of each coated hydrogel and coating thicknesses were measured via software analysis (MetaMorph, Molecular Devices, Sunnyvale, CA). A modified ELISA was employed to quantify the concentration of accessible IgG incorporated within the coatings, as we have previously described [18]. Briefly, IgG (from goat) was polymerized into coatings as described above and incubated overnight in PBS to ensure complete removal of unconjugated proteins. Coated gels were incubated for 30 min with donkey-anti-goat secondary IgG (horse-radish peroxidase (HRP)-conjugated, 5 $\mu\text{g/ml}$) in PBS with 0.5% BSA. Gels were rinsed 4 $\times$ with PBS and transferred to PBS with 0.5% BSA for an additional 30 min to remove unconjugated secondary IgGs. Gels were rinsed an additional 4 $\times$ in PBS and transferred to individual wells of a 96-well plate in 70 μl PBS. TMB ELISA substrate was added to each well for 60s with frequent pipetting to mix and subsequently transferred to H_2SO_4 (2M) to quench the reaction. The absorbance was read at 450 nm and compared to signals of a calibration curve generated by known concentrations of HRP-conjugated secondary IgG. For dual-color imaging, samples were stained per the modified ELISA, except FitC-IgG (from goat) was incorporated into polymer coatings and samples were incubated in rhodamine-conjugated donkey-anti-goat secondary IgG. Coated gels were imaged via confocal microscopy and z-direction images were constructed to visualize FitC-IgG throughout the entire coating and Rhod-IgG secondary IgG that bound accessible, polymerized IgGs.

2.7 T cell Apoptosis studies and flow cytometry

The biological activity of anti-fas-functionalized coatings was assessed via Jurkat T cell apoptosis studies. Coatings functionalized with thiolated anti-fas antibody (DX2 clone) and/or ICAM-1-Fc chimera were formed as described above and incubated overnight (>12 hrs) in catalase-containing media with at least 3 media changes. After 24 hrs, coated gels were placed in individual wells of a 96-well plate and 100 000 T cells / well were seeded atop coatings in 200 μl media. T cells were incubated for 12, 24, or 48 hours and then analyzed for apoptosis and necrosis via Vybrant #3 apoptosis assay. T cells were stained with FitC-Annexin V and propidium iodide (PI) to label apoptotic and dead cells, respectively. Cells were analyzed using a CyAn flow cytometer (Beckman Coulter, Brea, CA). T cells were considered apoptotic if they stained positive for FitC-Annexin V and negative for PI. All cells staining positive for PI were considered dead.

2.8 Cell encapsulation, coating & analysis

Min6 β -cells (15×10^6 cells/ml) or 3T3 fibroblasts (5×10^6 cells/ml) were encapsulated within PEG hydrogels by suspension in pre-polymer solutions containing PEGDA (10 kDa, 10 wt%), 1-[4-(2-hydroxyethoxy)phenyl]-2-hydroxy-2-methyl-1-propan-1-one (I2959, 0.05 wt%) as a photoinitiator, and catalase (100 nM). The peptide CRGDS (1mM) was also added when 3T3 cells were encapsulated to avoid anoikis. 30 μl of cell-containing pre-polymer suspensions were transferred into 1 ml syringe tips and photopolymerized for 10 min, under 6 mW/cm^2 UV light centered at 365 nm, yielding hydrogel disks of ~2 mm

thickness and ~5 mm in diameter. Cell-laden hydrogels were transferred to cell culture media and incubated overnight at 37°C. Coatings were formed via a GOx-mediated surface polymerization from the cell-laden gels as described above. Following the coating process, gels were transferred to cell culture media containing catalase (100 nM) and media was changed 3 times per hour for at least the first 2 hours following coating. Then, gels were incubated overnight at 37°C. Cell viability was assessed via a Live/Dead staining assay in which calcein AM labels live cells green and propidium iodide labels dead cells red. Cell-laden gels were imaged via confocal microscopy whereby 20 images were taken at 10 µm intervals and projected into a single plane. The CellTiter glo assay was used to quantify cellular viability as follows: Cell-laden hydrogels were transferred to 50% cell titer glo solution in PBS (200 µl /sample) and incubated 40 min with orbital shaking. 100 µl / sample was transferred to an opaque-walled, white 96-well plate and luminescence was recorded and compared to that of ATP standards.

2.9 Statistical Analysis

Results presented herein are expressed as mean ± standard error of the mean. Two-tailed, unpaired Student's t-tests were used to determine statistical significance between data sets. Differences were considered statistically significant when the p value was less than 0.05. All experiments were replicated at least 3 times.

3. RESULTS

3.1 Glucose oxidase-mediated polymer coatings

The GOx enzyme was utilized to initiate polymerization on the surfaces of hydrogels as described above and illustrated in Figs. 1A–C. Uniform coatings, such as the one shown in Fig. 1D, were polymerized on the surfaces of PEG hydrogels by dipping glucose-swollen hydrogels into glucose-free pre-polymer containing GOx enzyme, Fe²⁺ and either PEG monoacrylate (acryl-PEG) or PEG monomethacrylate (methacryl-PEG). Coating thicknesses were controlled by varying the dipping time, and a pseudo-linear relationship was observed between dipping time and the resulting coating thickness, as shown in Fig. 2. Coating thicknesses up to 450±50 µm were fabricated for dipping times ranging 0 to 60 s. As shown in Fig. 2, conformal coatings are formed using either acryl-PEG or methacryl-PEG. Further, 3D visualization via confocal microscopy confirmed that a conformal layer is formed on all faces of the hydrogel.

3.2 Incorporating proteins & signaling molecules into polymer coatings

IgGs, anti-Fas, and ICAM-1 were rendered polymerizable via the addition of thiol groups to free amines by reaction with Traut's reagent. The extent of protein modification was controlled by varying the molar excess of Traut's reagent relative to protein. Protein modification was confirmed in two different manners: the appearance of thiol groups on purified protein was monitored via Ellman's reagent, and the disappearance of free amines was observed using Fluoraldehyde reagent. Both measures confirmed that the extent of thiolation was proportional to the molar excess of Traut's reagent (data not shown). Once thiolated, IgG-SH, anti-Fas-SH and ICAM-1-SH were incorporated into polymer coatings via GOx-initiated thiol-acrylate polymerization, as we have previously demonstrated that proteins and peptides containing free thiol groups may be incorporated into polymer networks via mixed mode thiol-acrylate polymerizations [34]. Briefly, the thiol acts as a chain transfer agent, having its hydrogen abstracted by the propagating radical of the growing (meth)acrylic coating. The new thiyl radical that is formed reinitiates a new (meth)acrylic polymer chain which, due to the multifunctional nature of the (meth)acrylate monomers, becomes crosslinked into the original coating, thus uniformly incorporating the protein into the coating.

3.3 Characterization of proteins & signaling molecules within polymer coatings

A previously reported modified ELISA was utilized to detect available proteins incorporated within the polymer network [18]. Thiolated IgGs from goat were incorporated at concentrations ranging from 0 to 250 $\mu\text{g/ml}$ into polymer coatings and incubated with donkey anti-goat secondary antibodies with conjugated HRP or fluorophore to detect or visualize, respectively, IgGs incorporated within polymer coatings. FitC-labeled IgG-SH was polymerized into polymer coatings and then incubated with rhodamine-conjugated secondary IgG. As shown in Fig 3A, a green polymer coating containing FitC-IgG was visible on both faces of a coated hydrogel. Red secondary IgG (IgG-Rhod) was incubated with coatings and staining was most evident at the surface of the coating. Some staining with secondary IgG was visible within the polymer coatings (indicated by yellow). Control coatings lacking FitC-IgG-SH were fabricated and exposed to IgG-Rhod secondary IgG and no green or red staining was visible (data not shown), indicating that the secondary IgG staining in Fig 3A was specific for IgGs polymerized into the coating.

To quantify the amount of IgG-SH within coatings that remained accessible to the surroundings, samples were incubated with HRP-conjugated secondary IgG, reacted with TMB ELISA substrate, and the substrate absorbance change was compared to a standard curve [18]. The amount of detectable IgG was found to increase as the concentration of IgG-SH in the coating pre-polymer solution was increased, Fig. 3B, and surface concentrations in excess of 10 ng IgG per coated cm^2 were achieved. The amount of detectable IgG as a function of coating formation time was also analyzed, Fig. 3C. For the first 20 s of coating, the amount of detectable IgG increased with time; however, the amount of detectable IgG remained constant for longer polymerization times, Fig 3C. This relationship suggests that IgGs were accessible to secondary IgG within approximately the outermost 100 – 150 μm of the coating (the thicknesses achieved for coating times of 20 s or 30 s for acryl-PEG or methacryl-PEG, respectively). While the overall coating thickness increases for longer reaction times, it is likely that the additional polymer formation of a thicker coating limits the accessibility of IgG near the original surface. Thus, a relatively constant amount of IgG-SH remained accessible to the surroundings for coating times in excess of ~20 to 30 s.

3.4 Inducing T cell apoptosis with functionalized dip coatings

Thiolated anti-Fas and ICAM-1 were incorporated into GOx-initiated polymer dip-coatings as described above. A 30 s reaction time was used for all T cell studies to allow a high concentration of accessible protein. Coated hydrogels were swollen overnight in catalase-containing cell culture media to ensure removal of unconjugated signaling proteins. The following day, Jurkat T cells were seeded atop functionalized coatings and cultured for 24 hrs. Following 24 hrs, T cells were harvested by PBS rinse and analyzed for apoptosis by FitC-Annexin V staining and flow cytometry. Samples were counter-stained with propidium iodide to label dead cells so that only live cells were included in analysis. Flow cytometry of T cells 24 hrs following seeding atop functionalized coatings revealed significant apoptosis when cultured on coatings containing co-polymerized anti-fas, Figs. 4A & 4B. As shown in Fig. 4B, for T cells seeded atop blank or ICAM-1-only coatings, a low level of apoptosis was observed, $2\pm1\%$ and $4\pm2\%$ (for PEG acrylate-based coatings), respectively. Coatings functionalized with anti-fas induced significantly higher levels of apoptosis, $13\pm2\%$. Coatings fabricated with acryl-PEG that were dually-functionalized with anti-Fas and the ICAM-1 adhesion ligand induced the highest percentage of T cell apoptosis, $22\pm2\%$.

3.5 Apoptosis time course

To assess the impact that functionalized coatings have on the local T cell population over time, a 48 hr time course experiment was conducted. T cells seeded atop dually-functionalized (anti-Fas & ICAM-1) coatings fabricated with acryl-PEG were analyzed for

apoptosis and cell death, via staining and flow cytometry, at 12, 24, and 48 hrs. For T cells seeded atop blank coatings, Fig. 5A, a low level of apoptosis and cell death was observed for all time points. When seeded atop dually-functionalized (anti-Fas and ICAM-1) coatings, Fig. 5B, increased apoptosis was observed for all time points. The number of cells undergoing apoptosis increased from 12 to 24 hrs but remained similar from 24 hrs to 48 hrs. Interestingly, the number of dead cells increased dramatically from 24 to 48 hrs, from $17\pm2\%$ to $37\pm4\%$, respectively. This observation was attributed to the likelihood that cells undergoing apoptosis eventually die, and dead cells accumulate on the gel surface over time. Following seeding atop anti-fas / ICAM-1 coatings, apoptosis was monitored over 48 hrs, and $61\pm9\%$ of all cells were either apoptotic or dead, compared to only $18\pm5\%$ of T cells on non-functionalized coatings. This result indicates that anti-fas-functionalized polymer coatings formed via GOx-initiated surface-mediated polymerizations were able to significantly reduce the local T cell population in culture.

3.6 GOx coating of cell-laden hydrogels

A critical aspect of functionalizing cell-laden carriers is the cytocompatibility of the coating process. To assess the cytocompatibility of coatings formed via GOx-mediated polymerization on cell-laden hydrogels, Min6 β -cells and 3T3 fibroblasts were encapsulated in PEG hydrogels and then reactively dip-coated. Interestingly, low molecular weight PEG acrylates ($M_n = 400$ Da) were found to be highly toxic to encapsulated cells, show in Fig. 6, even at the shortest coating times. When immersed in low molecular weight PEG methacrylates ($M_n = 525$ Da), however, high cell viability was maintained. Viability was assessed 24 hrs following GOx-mediated coating via live/dead staining and confocal microscopy as well as ATP viability assay. As shown in Fig. 6A, high cell viability was visualized for both Min6 and 3T3-laden hydrogels dipped for 30s in methacryl-PEG-containing pre-polymer solutions containing either 0 or 50 μ M GOx enzyme. Of note, immersion in the 50 μ M GOx solution resulted in significant coating formation. Hydrogels containing either Min6 β -cells (Fig. 6B) or 3T3 fibroblasts (Fig. 6C) were coated via dipping in acryl-PEG or methacryl-PEG and subsequently cultured in catalase-containing cell culture media for 24 hrs before viability was quantified. As shown in Figs. 6B & C, high cell viability remained after 24 hrs for coatings fabricated with methacryl-PEG, but essentially no viability remained after dipping in acryl-PEG.

4. DISCUSSION

Strategies to form multi-layer hydrogel structures offer the ability to spatially regulate biological and cellular functions within biomaterials systems. Here, glucose-swollen hydrogels were dipped into pre-polymer solutions containing the glucose oxidase enzyme to form conformal coatings on cell-laden structures and introduce biologically relevant functionalities that manipulate the local immune response. While conceptually straightforward, two critical design parameters relate to the thickness and bioactivity of the coating, combined with the cytocompatibility of the processing conditions. Many concentrations of GOx enzyme and Fe^{2+} were investigated (data not shown), but concentrations of 50 μ M and 4 mM, respectively, were used for all reported studies, unless otherwise stated, as they enabled the rapid formation (0 to 60 s) of coatings (approximately 0 to 500 μ m). The thicknesses of fluorescently-labeled coatings were measured via confocal microscopy and a pseudo-linear relationship was found between immersion times and coating thickness, Fig. 2. This relationship agreed well with that previously observed by Johnson *et al.* [32]. PEG monoacrylate (acryl-PEG, $M_n = 400$ Da) and PEG monomethacrylate (methacryl-PEG, $M_n = 525$ Da) were used as the base monomers for coatings in this study because we have previously demonstrated immune-cell signaling by functionalized coatings fabricated from monoacrylated PEGs [18]. While mono-(meth)acrylated PEGs were

evaluated herein, di(meth)acrylated PEGs have also been shown to be suitable for GOx-mediated dip coatings [32].

Proteins were rendered reactive for copolymerization via covalent modification with free thiol groups. As previously investigated in our laboratory, thiolated peptides and proteins may be covalently conjugated into polymer networks via radical-mediated thiol-acrylate polymerizations [34, 35]. Thiolated IgGs were incorporated into coatings atop the surfaces of hydrogels via GOx-mediated dip coatings. Incorporation was verified by fluorescent confocal microscopy and ELISA-type assays, Fig. 3. High concentrations of proteins were detectable within polymer coatings, exceeding 10 ng per coated cm² of hydrogel. These concentrations were over 7-fold greater than those that we have previously observed for functionalized, polymeric coatings fabricated via surface-initiated photopolymerizations [18]. This increase in detectable, incorporated IgG is likely due to the increased coating thickness and the higher density of initiating sites that are formed by the GOx-mediated polymerization. Previous coating thicknesses achieved by grafted photoinitiators ranged from 0 to approximately 50 μ m [18].

To signal immune cells in proximity to hydrogels, thiolated anti-fas IgG was incorporated within coatings. We previously investigated the induction of T cell apoptosis by PEG-based polymer coatings and observed that significant apoptosis was induced by surfaces functionalized with anti-fas IgG [18]. Additionally, we determined that incorporating the ICAM-1 adhesion protein further improved fas signaling [18], likely by increasing the interaction between T cells and the PEG surface [36] and promoting fas signaling [37]. Because of these previous findings, we chose to incorporate anti-fas IgG and ICAM-1 into the GOx-mediated PEG coatings investigated herein. Jurkat T cell studies verified that anti-fas retained significant bioactivity when incorporated into coatings, as apoptosis was successfully induced. When analyzed for apoptosis at 12, 24 and 48 hrs, it was clear that the dually-functionalized anti-Fas / ICAM-1 coatings had a significant impact on the local T cell population. After 48 hrs atop functionalized coatings, less than 40% of cells remained healthy.

To verify the cytocompatibility of this coating process, cell-laden hydrogels were coated and subsequently cultured overnight. When dipped in pre-polymer solutions containing acryl-PEG, interestingly, poor cell viability was observed. In fact, in the absence of any other factors, dipping in 10 wt% acryl-PEG ($M_n=400$ Da) for very short times (e.g., 10 s) was sufficient to result in almost complete cell death to encapsulated cells (data not shown). Coating with methacryl-PEG, however, was better-tolerated by encapsulated cells and resulted in high viability after 24 hrs. Because higher surface concentrations of detectable signaling molecules were observed on hydrogels fabricated with acryl-PEG, this coating formulation may be superior to methacryl-PEG-based coatings for the singular purpose of signaling nearby cells. If it is desirable to coat cell-laden hydrogels, however, methacryl-PEG-based coatings are clearly superior due to drastically-improved cytocompatibility of the compounds evaluated here.

Following 24 hrs, coated gels retained high viability when cultured in media containing catalase. In the absence of catalase, however, low cell viability was observed. Because the addition of catalase, a commercially available enzyme which breaks down H₂O₂, was sufficient to provide high cell viability, H₂O₂ formation likely continued after dip-coating. This observation might be attributed to the encapsulation of GOx enzyme within the polymer coatings, which may subsequently continue to react with glucose in cell culture media. Once entrapped with PEG coatings, the large (160 kDa [38]) GOx molecule is unlikely to diffuse out of the PEG network [39]. In the presence of catalase, however, high

β -cell and 3T3 viability were observed. Ultimately, as previously described [33], the glucose oxidase chemistry can be highly cytocompatible.

5. CONCLUSIONS

We have demonstrated a cytocompatible dip-coating technique for modifying cell-laden hydrogels and for the purpose of fabricating immunoactive coatings. GOx enzyme allows for the initiation and formation of uniform, PEG-based polymer coatings with thicknesses controlled via the reaction/dipping time. Thiol-acrylate polymerization enabled thiolated biomolecules to be covalently and efficiently incorporated into the coatings, and a modified ELISA verified that a high density of incorporated protein was accessible to the surroundings. To form an immunoactive coating around PEG hydrogels, anti-fas antibody and ICAM-1 adhesion molecule were incorporated into the surface layer. Studies with T cells verified that functionalized coatings induced significant fas signaling, as over 60% of all T cells were apoptotic or dead after 48 hrs atop the coatings. Finally, the cytocompatibility of methacryl-PEG-based coatings was studied and verified in the presence of catalase. In summary, GOx-mediated dip coatings are an attractive technique for functionalizing cell-laden biomaterials with biologically active molecules to signal external cells, including immune cells.

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Fig. 1.

Schematic illustrating the formation of polymer coatings initiated by glucose oxidase (GOx). (A) Cell-laden PEG hydrogels are swollen in a glucose-containing media and then (B) dipped into a pre-polymer solution containing acryl-PEG, GOx, Fe^{2+} , and thiolated signaling molecules. Glucose diffuses out of the gel, reacts with GOx and initiates polymerization at the surface of the hydrogel. (C) Reactive coating results in conformal PEG layers. Schematic not to scale. (D) Confocal micrograph of PEG hydrogel (green) with GOx-mediated polymer coating (red). Scale = 200 μm .

Fig. 2. Polymer coating thickness as a function of dipping-time. Fluorescent molecules were incorporated within polymer coatings and then visualized and measured via confocal microscopy. Coatings were formed from dipping solutions containing 25 wt% of either acryl-PEG (black line) or methacryl-4 μ M Fe^{2+} .

Fig. 3.

Incorporation of biomolecules within polymer coatings. (A) z-stack confocal image of coatings on the top and bottom of a PEG hydrogel. FitC-labeled goat IgG was covalently incorporated within polymer coating (green). When incubated with rhodamine-conjugated donkey-anti-goat IgG, accessible goat IgG is fluorescently labeled (red). For simplicity, illustration shows coatings only on top and bottom of the hydrogel; actual samples were coated on all sides. (B) Concentration of accessible IgG in polymer coatings, per cm² of hydrogel coated, as a function of the concentration of IgG-SH loaded into the pre-polymer. (C) Concentration of accessible IgG in polymer coating as a function of dip-coating time. Coatings were formed with 25 wt% of either acryl-PEG (black line) or methacryl-PEG (dashed red line) with 50 μ M GOx and 4 mM Fe²⁺.

Fig. 4.

Induction of T cell apoptosis atop functionalized PEG coatings. (A) Flow cytometry of T cells harvested from functionalized coatings fabricated with acryl-PEG. Live cells (PI neg) stained with FitC-Annexin V are shown and the percentage of cells positive for apoptosis increased with the incorporation of anti-fas and anti-fas/ICAM-1. (B) Summary of findings for functionalized coatings composed of 25 wt% of either acryl-PEG (solid bar) or methacryl-PEG (slashed red bar). * denotes $p < 0.05$ difference. \$ denotes $p < 0.05$ difference from -/- acryl-PEG control. # denotes $p < 0.05$ difference from -/- methacryl-PEG control.

Fig. 5.

T cells were seeded atop control (A) or dually-functionalized (anti-Fas + ICAM-1) (B) coatings composed of 25 wt% acryl-PEG for 12, 24, or 48 hrs and then analyzed via flow cytometry. The fraction of dead and apoptotic cells for each condition was measured. Functionalized coatings induced a significant increase in the number of T cells undergoing apoptosis.

Fig. 6.

Cytocompatibility of GOx-initiated polymer coatings toward encapsulated cells (A) Live/dead confocal microscopy of cell-laden hydrogels containing (top) Min6 β -cells or (bottom) 3T3 fibroblasts. Samples were dipped for 30 s in a pre-polymer solution containing (left) 0 μ M or (right) 50 μ M GOx enzyme with 25 wt% methacryl-PEG and then analyzed at 24 hrs. The 50 μ M GOx condition resulted in coating formation. Green and red staining denotes live and dead cells, respectively. Scale = 100 μ m. (B–C) Viability of encapsulated Min6 β -cells (B) or 3T3 fibroblasts (C) 24 hours after coating. Coatings were fabricated with 25 wt% of either acryl-PEG acrylate (solid bar) or methacryl-PEG (slashed red bar). Virtually no metabolic activity remained after 24 hrs with hydrogels dipped in acryl-PEG coating solutions.